MCP-1 impacts RCT by repressing ABCA1, ABCG1, and SR-BI through PI3K/Akt posttranslational regulation in HepG2 cells

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Abstract  Monocyte chemoattractant protein-1 (MCP-1) plays crucial roles at multiple stages of atherosclerosis. We hypothesized that MCP-1 might impair the reverse cholesterol transport (RCT) capacity of HepG2 cells by decreasing the cell-surface protein expression of ATP binding cassette A1 (ABCA1), ATP binding cassette G1 (ABCG1), and scavenger receptor class B type I (SR-BI). MCP-1 reduced the total protein and mRNA levels of ABCA1 and SR-BI, but not of ABCG1. MCP-1 decreased the cell-surface protein expression of ABCA1, ABCG1, and SR-BI in dose-dependent and time-dependent manners, as measured using cell-surface biotinylation. We further studied the phosphoinositide 3-kinase (PI3K)/serine/threonine protein kinase Akt pathway in regulating receptor trafficking. Both the translation and transcription of ABCA1, ABCG1, and SR-BI were not found to be regulated by the PI3K/Akt pathway. However, the cell-surface protein expression of ABCA1, ABCG1, and SR-BI could be regulated by PI3K activity, and PI3K activation corrected the MCP-1-induced decreases in the cell-surface protein expression of ABCA1, ABCG1, and SR-BI. Moreover, we found that MCP-1 decreased the lipid uptake by HepG2 cells and the ABCA1-mediated cholesterol efflux to apoA-I, which could be reversed by PI3K activation. Our data suggest that MCP-1 impairs RCT activity in HepG2 cells by a PI3K/Akt-mediated posttranslational regulation of ABCA1, ABCG1, and SR-BI cell-surface expression.

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Atherosclerosis results from an excessive proliferative and inflammatory response in the vascular wall (1). Monocyte chemoattractant protein-1 (MCP-1)/CCL2, a member of the CC chemokine family, is thought to be most strongly implicated in the initiation and progression of atherosclerosis (2–4). MCP-1/CCL2-knockout mice placed on an LDL receptor-deficient background showed a significant reduction in atherosclerotic plaques (5). Several large cohort studies (6, 7) show that MCP-1 may mediate the proatherogenic effects of dyslipidemia and is therefore a potential therapeutic target. A low plasma high density lipoprotein (HDL) cholesterol level is recognized as a major independent risk factor for the development of coronary heart disease (CHD) (8). However, HDL possesses key atheroprotective biological properties (9, 10), and the most important one is thought to be its ability to remove excess cholesterol from peripheral tissues then deliver it to the liver for biliary excretion by a process called reverse cholesterol transport (RCT) (11–13). Recently, large cohort studies (14, 15) demonstrate that the cholesterol efflux capacity can act as another potential measure of CHD risk assessment. The HDL receptors, ATP binding cassette A1 (ABCA1), ATP binding cassette G1 (ABCG1), and scavenger receptor class B type I (SR-BI) play crucial roles in RCT and have been found to be expressed not only on the plasma membrane but also in intracellular vesicles (16–18). Researchers have indicated that the localization and cell-surface expression of ABCA1, ABCG1, and SR-BI can be modulated by many substances and then in turn affect the RCT activity (18–20).

The activity of RCT, an HDL-mediated atheroprotective biological property, is impaired during inflammatory...
states. The acute-phase response, which can be induced by infection or inflammation, impairs the capacity of the human HUH-7 hepatoma cell line to deliver cholesteryl ester and diminishes the cholesterol efflux capacity of macrophages (21, 22). Irina et al. (23) demonstrated that lipopolysaccharide treatment resulted in the downregulation of SR-BI and ABCA1 expression, a significant inhibition of HDL-mediated cholesterol efflux, compared with untreated RAW 264.7 cells. Many pro-inflammatory factors, including IFN-γ, lipopolysaccharide, tumor necrosis factor, interleukin-1, and interleukin-6, have been found to modulate the expression of HDL receptors and, in turn, alter RCT activity (24–26).

However, the impact of MCP-1 on HDL-mediated RCT activity and whether this effect contributes to the expression or redistribution of the relevant transporters have not been investigated. In this report, we used the HepG2 cell line to provide evidence that the pro-atherogenic effects of MCP-1 may reflect, at least in part, altered cholesterol metabolism through RCT. We then focused on the involvement of the phosphoinositide 3-kinase (PI3K)/serine/threonine protein kinase Akt pathway, which posttranslationally regulates the recruitment of receptors to the plasma membrane.

MATERIALS AND METHODS

Cell culture and treatment

The HepG2 cells were a generous gift from the medical school of Sun Yat-sen University. The cells were grown in Dulbecco’s modified Eagle medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C. The cells were grown until 60–70% confluent and were then incubated in a serum-free medium containing 0.5% BSA for 6 h. To investigate the dose effect of MCP-1, cells were incubated in a serum-free medium containing 0.5% BSA with MCP-1 (R and D Systems, Inc., Abingdon, UK) at different concentrations (0–80 ng/ml) for 48 h. Cells treated with MCP-1 at 40 ng/ml for increasing times (0–72 h) were used to study the time effect of MCP-1. To further examine the involvement of PI3K, we first pretreated the cells with either the PI3K activator (insulin) or a PI3K inhibitor wortmannin (100 nM; Cell Signaling Technology, Beverly, MA) for 45 min. The cells were then incubated with or without MCP-1 (40 ng/ml) for 48 h.

Western blotting

Following the incubation, the cells were harvested, washed with phosphate-buffered saline (PBS) (pH 7.4), and lysed in RIPA buffer (Roche Molecular Biochemicals) for 30 min at 4°C. The proteins were fractionated on 4–10% gradient SDS/polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA). The membranes were incubated with a TBS blocking solution (200 mM Tris-HCl, 150 mM NaCl, 5% nonfat dry milk) for 1 h at room temperature. The membranes were immunoblotted with the appropriate antibody: mouse monoclonal anti-ABC1 antibody (Novus Biologicals, Oakville, CA; diluted 1:1,000), rabbit polyclonal anti-ABCG1 antibody (Novus Biologicals; diluted 1:1,000), goat polyclonal anti-SR-BI antibody (Novus Biologicals; diluted 1:1,000), anti-Ser\textsuperscript{473}-phosphorylated Akt (p-Akt) (Cell Signaling Technology; diluted 1:750), anti-Akt (Cell Signaling Technology; diluted 1:750), or mouse monoclonal anti-β-actin antibody (Sigma; diluted 1:2,000) overnight at 4°C. After three washes with TBS containing 0.1% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. To visualize the immunoreactive bands, enhanced chemiluminescence (ECL) Western blotting detection reagents and medical X-ray films were used according to the manufacturer’s suggestions. The band intensity was analyzed with Quantity One. The data were normalized to the β-actin expression.

Real-time PCR

Total RNA was purified from the cultured cells using TRIzol (Invitrogen) according to the manufacturer’s protocol and treated with DNase I to remove any residual DNA contamination. First-strand cDNA synthesis was performed using a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara Bio, Japan) according to the manufacturer’s protocol. Real-time PCR was performed using a SYBR Green PCR Master Mix Kit (Applied Biosystems, Foster, CA). The gene expression of ABCG1, ABCA1, and SR-BI was normalized to that of β-actin. The primers used for quantitative RT-PCR are presented in supplementary Table I.

Confocal microscopy

To investigate the subcellular localization of the transporters, HepG2 cells cultured in chamber slides were washed, fixed, and permeabilized. The cells were then incubated with mouse anti-human ABCA1 antibody (1:25 dilution; Novus Biologicals), rabbit anti-human ABCG1 antibody (1:25 dilution; Novus Biologicals), or goat anti-human SR-BI antibody (1:25 dilution; Novus Biologicals). Subsequently, the cells were incubated with the following fluorescent secondary antibodies: Alexa Fluor\textregistered 546 Donkey Anti-Mouse IgG (Invitrogen), Alexa Fluor\textregistered 488 Donkey Anti-Rabbit IgG (Invitrogen), and Alexa Fluor\textregistered 633 Donkey Anti-Goat IgG (Invitrogen), respectively, for ABCA1, ABCG1, and SR-BI. After being washed with PBS, the cells were examined using confocal microscopy.

Cell-surface protein assays using biotinylation

For the cell-surface ABCA1, ABCG1, and SR-BI analyses, cells were first biotinylated with 0.5 mg/mL EZ-Link Sulfo-NHS-SS-Biotin (Pierce Chemical Co., Rockford, IL) at 4°C for 30 min. The cells were then lysed with RIPA buffer at 4°C. After centrifugation, the supernatants were incubated with anti-FLAG agarose beads overnight at 4°C. Following centrifugation and washing, the supernatants and pellets (the collected agarose beads) represented intracellular and surface proteins, respectively. The proteins were dissociated from the pellets by boiling with SDS loading buffer and were analyzed with SDS-PAGE and immunoblotting using mouse monoclonal anti-ABC1 antibody (Novus Biologicals; diluted 1:1,000), rabbit polyclonal anti-ABCG1 antibody (Novus Biologicals; diluted 1:1,000), goat polyclonal anti-SR-BI antibody (Novus Biologicals; diluted 1:1,000), or anti-Na\textsuperscript{+}/K\textsuperscript{+} ATPase antibody (Pierce Biotechnology; diluted 1:1,000).

1,1’-dioctadecyl-3,3,3’,3’-tetramethyldiocarbocyanine perchlorate (Dil)-HDL lipid uptake assay

Cells were grown on glass coverslips until 60–70% confluent and were then incubated in DMEM containing 0.5% BSA with or without MCP-1 (40 ng/ml) for 48 h following pretreatment with a PI3K activator (insulin) or a PI3K inhibitor (wortmannin) for 45 min. Dil-HDL (Biomedical Technologies Inc., Stoughton, MA) was added to a serum-free medium containing 0.5% BSA to obtain a final concentration of 5 μg/ml. After a 4 h incubation with Dil-HDL, the cells were fixed with 3% paraformaldehyde and washed with PBS. The cells were then incubated with horseradish peroxidase-conjugated secondary antibody in PBS at room temperature for 1 h. After washing with PBS, the cells were examined using confocal microscopy.
were washed with PBS, fixed, and subjected to laser confocal microscopy. For each experiment, all the pictures were identically exposed and processed.

**Cholesterol efflux from HepG2 cells**

HepG2 cells were seeded on collagen-coated 24-well plates at a density of 1 x 10^5 cells per well in DMEM supplemented with 10% FBS. After a 6 h serum starvation, the cells were washed with PBS and labeled by incubation in DMEM supplemented with 0.2% BSA containing [3H]cholesterol (0.5 uCi/ml; Perkin Elmer, CA) for 48 h. The cells were pretreated with a PI3K activator (insulin, 100 nM) or a PI3K inhibitor (wortmannin, 100 nM) for 45 min. Cellular cholesterol efflux was initiated by the addition of DMEM containing 0.2% BSA with 20 μg/ml human apoA1 in the presence or absence of MCP-1 (40 ng/ml). After a 48 h incubation, the radioactivity of the medium and cells was measured with a liquid scintillation counter. The cholesterol efflux was expressed as the percentage of counts in the medium relative to the total counts in the medium and cells together.

**Statistical analysis**

All the data were expressed as means ± SEM. The statistical significance of differences was determined using Student’s t-test or a one-way ANOVA followed by Bonferroni’s post hoc test, as appropriate. Statistical significance was defined as a two-tailed probability of less than 0.05.

**RESULTS**

**MCP-1 decreased the total protein expression of ABCA1 and SR-BI but did not affect the total protein expression of ABCG1 in HepG2 cells**

To investigate whether MCP-1 could alter the total protein expression levels of ABCA1, ABCG1, or SR-BI, we treated HepG2 cells for 48 h with increasing doses of MCP-1 (0–80 ng/ml) in the dose-effect study. We also treated HepG2 cells with MCP-1 at a fixed concentration of 40 ng/ml for increasing durations (0–72 h) in the time-course study. As shown in **Fig. 1A**, 40 ng/ml MCP-1 decreased the total protein expression of ABCA1 and SR-BI by up to 44 and 32%, respectively, compared with the untreated group. Fig. 1B shows the time-course responses of the ABCA1, SR-BI, and ABCG1 protein expression to 40 ng/ml MCP-1. The MCP-1 treatment resulted in a significant inhibition (79%) of the ABCA1 expression at 72 h compared with the untreated cells. In addition, the SR-BI expression was reduced to 44% of the control level at 48 h. However, the MCP-1 intervention did not alter the total protein expression of ABCG1. Furthermore, mouse primary hepatocytes were also incubated with MCP-1 (40 ng/ml) for 48 h to test the effects of MCP-1 on mouse primary hepatocytes, and similar results are shown in supplementary Fig. II.

**MCP-1 decreased the mRNA expression of ABCA1 and SR-BI but did not alter the mRNA expression of ABCG1**

We next studied whether MCP-1 could induce the gene expression of the three receptors. HepG2 cells were treated in the same manner as for the total protein detection. As shown in **Fig. 2A**, MCP-1 repressed the gene expression of both ABCA1 (at 40 ng/ml) and SR-BI (at 80 ng/ml) by approximately 80% in the dose-effect group. Fig. 2B shows the time-effect response to MCP-1 of the gene expression of the three acceptors. Treatment with MCP-1 (40ng/ml) for 48 h resulted in significant decreases in the mRNA levels of ABCA1 and SR-BI, to 17 and 48%, respectively, of the levels of the untreated cells. The addition of MCP-1 did not alter the ABCG1 mRNA levels. Most importantly, the treatment of the HepG2 cells with MCP-1 repressed the ABCA1 and SR-BI mRNA levels with no changes in the ABCG1 gene expression, which was similar to the effects of MCP-1 on the total protein levels of the three genes. The impaction of MCP-1 on mouse primary hepatocytes ABCA1, ABCG1, and SR-B1 mRNA (supplementary Fig. II) were similar to that in HepG2 cells.

**The subcellular localization and cell-surface protein expression of ABCA1, SR-BI, and ABCG1 in HepG2 were regulated by MCP-1**

Many studies have indicated that the subcellular localization of ABCA1, ABCG1, and SR-BI can be posttranslationally modulated by certain substances to allow them to move to the plasma membrane and, in turn, affect RCT (18–20, 27). We have shown that treatment with MCP-1 resulted in reductions in the ABCA1 and SR-BI total proteins while leaving the ABCG1 level unchanged. However, it was unknown whether MCP-1 could cause corresponding reductions in the cell-surface levels of the ABCA1 and SR-BI receptors, or could even affect the ABCG1 surface expression. The numbers of cell-surface receptors were directly measured using cell-surface biotinylation. As shown in **Fig. 3A**, the cell-surface expression levels of ABCA1 and SR-BI were reduced in a dose-dependent and time-dependent manner by up to 87 and 75%, respectively, after treatment with 40 ng/ml MCP-1 for 72 h. This result was not completely parallel to the changes in total protein expression. The cell-surface ABCG1 level of the HepG2 cells incubated with MCP-1 at 80 ng/ml for 48 h also decreased to 14% of the level of the untreated cells.

To confirm the distribution of the receptors in the HepG2 cells treated with MCP-1, the cells were examined under confocal microscopy. After reaching 60–70% confluence, the cells were equilibrated for 6 h and incubated in a serum-free medium in the presence or absence of MCP-1 (40 ng/ml), or 48 h. As shown in **Fig. 3B**, ABCA1, ABCG1, and SR-BI were found to be distributed throughout the cytoplasm and cell surface in the untreated cells. We found that the cell-surface proteins of ABCA1 and SR-BI were inhibited by MCP-1, which was in line with the changes in the numbers of cell-surface receptors. Strikingly, a 48 h incubation with 40 ng/ml MCP-1 resulted in both a marked redistribution of ABCG1 to the cell nucleus and a decreased distribution at the cell surface. This finding is in accordance with the change in the numbers of the cell-surface ABCG1 receptor.

**The Ser^473-phosphorylated Akt was regulated after treatment with MCP-1 in HepG2 cells**

Because PI3K plays a key role in cell transporter trafficking, we explored whether MCP-1 could alter PI3K/Akt
MCP-1 reduced the total protein and gene expression of ABCA1 and SR-BI without PI3K involvement

To further examine the regulation of the ABCA1, ABCG1, and SR-BI receptors by PI3K, we assessed the effects of PI3K activity on the total protein expression and mRNA levels of ABCA1, ABCG1, and SR-BI. Cells were treated with a PI3K activator (insulin) or inhibitor (wortmannin) for 45 min. The cells were then incubated with or without MCP-1 (40 ng/ml) for 48 h. As shown in Fig. 5, the expression of p-Akt was induced by the PI3K activation mediated by insulin. In contrast, p-Akt was not detected when the cells were treated with the PI3K inhibitor, either in the absence or presence of insulin. In the cells that were co-incubated in a medium containing insulin (100 nM) and MCP-1 (40 ng/ml) for 45 min, p-Akt was repressed; specifically, MCP-1 repressed the insulin-induced p-Akt. This phenomenon suggests that MCP-1 downregulates PI3K/Akt activity.

Fig. 1. The effects of MCP-1 on the protein expression of ABCA1, SR-BI, and ABCG1 in HepG2 cells. Starved HepG2 cells were treated with either increasing concentrations of MCP-1 (0–80 ng/ml) in DMEM containing 0.5% BSA for 48 h (A) or with MCP-1 at 40 ng/ml (B) for the indicated times. Total proteins were extracted from the cultured cells, and the protein levels were analyzed using Western blot analysis, as described in the Materials and Methods. The relative expression of ABCA1, ABCG1, and SR-BI was expressed as the ratios of ABCA1, ABCG1, and SR-BI to the corresponding β-actin expression. The error bars indicate the standard deviations. Each experiment was performed three times. ***P < 0.001, **P < 0.01, *P < 0.05 compared with the untreated group. The upper panel shows a Western blot and is representative of one experiment.

Fig. 2. The effects of MCP-1 on ABCA1, ABCG1, and SR-BI mRNA expression in HepG2 cells. HepG2 cells were treated with increasing concentrations of MCP-1 (0–80 ng/ml) in DMEM containing 0.5% BSA for 48 h (A) or with a fixed concentration of MCP-1 (40 ng/ml) (B) for the indicated times. RNA was extracted from the cultured cells, and the mRNA levels were analyzed with real-time PCR, as described in the Materials and Methods. The average copy numbers of ABCA1, ABCG1, and SR-BI were normalized to the β-actin expression. The results are expressed as fold inductions compared with the untreated controls (Cont.) ± SEM. **P < 0.01, *P < 0.05 compared with the untreated group. Each experiment was performed in triplicate.
To assess whether PI3K activity affects the subcellular localization of the three acceptors, the surface receptor levels were measured using cell-surface biotinylation. The biotinylation was performed after the treatment with MCP-1 and the following PI3K activation (by insulin) or inhibition (by wortmannin). The results in Fig. 6 show that PI3K activation significantly increased the cell-surface expression of ABCA1, ABCG1, and SR-BI. In contrast, PI3K inhibition, in both the absence and the presence of insulin, markedly decreased the numbers of receptors at the cell surface. PI3K activation with insulin could restore the numbers of ABCA1, ABCG1, and SR-BI receptors at the cell surface following treatment with MCP-1.

MCP-1 decreased Dil-HDL lipid uptake, which could be reversed by PI3K activation

Hepatocytes play a pivotal role in RCT, especially in SR-BI-mediated lipid uptake. Because inhibitions in the expression of total and cell-surface SR-BI proteins were observed in HepG2 cells after MCP-1 treatment, we assessed whether incubation with MCP-1 could alter Dil-HDL lipid uptake by HepG2 cells. We examined lipid uptake by incubating cells with Dil-HDL for 4 h following treatment with 40 ng/ml MCP-1 for 48 h. The cells were then subjected to confocal microscopy after fixation. Following incubation with MCP-1, cells had a greatly impaired lipid uptake capacity (red in Fig. 7B) compared with the cells with no MCP-1 treatment, as shown in Fig. 7A. To assess the ability of PI3K activity to influence Dil-HDL lipid uptake, cells pretreated with insulin or wortmannin for 45 min were incubated in the presence or absence of MCP-1 (40 ng/ml) for 48 h. As indicated in Fig. 7E, PI3K activation increased the Dil-HDL lipid uptake compared with the untreated controls. In contrast, PI3K inhibition, in both the absence (Fig. 7C) and the presence (Fig. 7G) of insulin, markedly decreased the lipid uptake. Pretreatment with...
insulin (Fig. 7F) improved the lipid uptake compared with the MCP-1 group (Fig. 7B).

MCP-1 decreased cholesterol efflux to apoA-I from HepG2 cells, which could be reversed by PI3K activation, and also reduced cholesterol efflux to apoA-I from mouse primary hepatocytes in an ABCA1-dependent manner.

Cholesterol efflux from hepatocytes via HDL transporters, namely, ABCA1, ABCG1, and SR-BI, or diffusional efflux is an important step for HDL formation. Based on the finding that MCP-1 reduced the expression and cell localization of the HDL transporters, we then investigated the effects of MCP-1 on the cholesterol efflux to apoA-I. After treating HepG2 cells with MCP-1 (40 ng/ml), we detected the cholesterol efflux to apoA-I, as indicated in the Materials and Methods. As shown in Fig. 8A, the cholesterol efflux to apoA-I in cells treated with MCP-1 was reduced by 54% compared with the untreated cells. However, whether Fig. 6. PI3K activation corrected the MCP-1-induced decreases in the numbers of the ABCA1, ABCG1, and SR-BI cell-surface receptors. HepG2 cells were equilibrated for 6 h in DMEM containing 0.5% BSA after reaching 60–70% confluence. The cells were then treated with (+) or without (−) MCP-1 after incubating with (+) or without (−) PI3K activation (by insulin) or inhibition (by wortmannin). The ABCA1, ABCG1, and SR-BI cell-surface receptor levels and Na+/K+ ATPase were directly extracted using cell-surface biotinylation and then measured by Western blotting, as described in the Materials and Methods. *P < 0.05 compared with the untreated group; ‡P < 0.05 compared with MCP-1 group.
MCP-1 impaired RCT in HepG2 cells

The knockdown of ABCA1 by si-ABCA1 resulted in a 52% reduction in the cholesterol efflux to apoA-I, whereas the knockdown of SR-BI resulted in a slight but significant increase in the cholesterol efflux. In addition, the knockdown of ABCG1 had little effect on the cholesterol efflux. The treatment with MCP-1 in hepatocytes transfected with si-ABCG1 or si-SR-BI still caused significant reductions in the cholesterol efflux compared with the cells that were not treated with MCP-1, whereas MCP-1 had no impact on the cholesterol efflux in mouse primary hepatocytes that were transfected with si-ABCA1 compared with the non-MCP-1 treatment. These results suggest that MCP-1 reduces the cholesterol efflux by decreasing the expression of the transporter ABCA1.

Because hepatocyte-secreted extracellular apoA-I is another determinant of cholesterol efflux, we detected the impact of MCP-1 on the apoA-I mRNA levels and secretion. As shown in supplementary Fig. I, MCP-1 did not affect either the level of apoA-I mRNA or the extracellular secretion of apoA-I, suggesting that the alteration in the cholesterol efflux to apoA-I is not affected by apoA-I secretion but is primarily affected by the amount of ABCA1 localized to the cell surface.

DISCUSSION

MCP-1 causes cholesterol accumulation in hepatocytes (28), and HDL receptors in hepatocytes contribute to cholesterol metabolism through the RCT process, including the generation of HDL and cholesterol ester uptake from HDL-cholesterol. Thus, we evaluated the effects of MCP-1 on the activities of HDL receptors in hepatocytes and the...
**Methods.** The values shown are the means ± SEM of triplicate.

**Results.**

Fig. 8. The effects of MCP-1 and PI3K activity on cholesterol efflux from HepG2 cells and the effects of MCP-1 and knockdown of ABCA1, ABCG1, and SR-BI on cholesterol efflux from mouse primary hepatocytes. HepG2 cells (A) or mouse primary hepatocytes (B) on collagen-coated 24-well plates were loaded with [3H]cholesterol (1 μCi/ml) for 48 h. After being washed with PBS, HepG2 cells were pretreated with (+) or without (−) the PI3K inhibitor wortmannin (100 nM) or activator insulin (100 nM) for 45 min and then incubated with or without MCP-1 (40 ng/ml) for 48 h, while mouse primary hepatocytes (Normal) or mouse primary hepatocytes transfected with si-ABCA1, si-ABCG1, si-SR-BI, or si-negative control (NC) were incubated with or without MCP-1 (40 ng/ml) for 48 h. The cholesterol efflux was initiated by the addition of Dulbecco’s modified Eagle medium containing 0.2% BSA with 20 μg/ml human lipid-free apoA-I. After a 48 h incubation, the cholesterol efflux was tested as mentioned in the Materials and Methods. The values shown are the means ± SEM of triplicate. ***P < 0.005, *P < 0.05, ns, not significant.

RCT capacity, and we selected the HepG2 cell line for these experiments in the present study. These data suggest that MCP-1 suppresses hepatic ABCA1 and SR-BI expression both transcriptionally and posttranslationally but only decreases ABCG1 cell-surface expression posttranslationally. We also further demonstrated the similar effects of MCP-1 on mouse primary hepatocytes (supplementary Fig. II). The PI3K/Akt pathway participates in the MCP-1-mediated posttranslational suppression of the cell-surface localization of ABCA1, ABCG1, and SR-BI, and the PI3K activator restores the impaired RCT activity caused by MCP-1.

The efflux of cholesterol to apoA-I is the first stage of biogenesis of HDL in hepatocytes (29). Several different potential cellular cholesterol efflux pathways have been described: diffusional efflux, ABCA1-, ABCG1-, and SR-BI-mediated cholesterol efflux pathways (30). The experiments in which mouse primary hepatocytes were transfected with siRNA indicated that the knockdown of ABCA1 induced a significant reduction in cholesterol efflux to apoA-I, whereas the knockdown of ABCG1 did not affect the cholesterol efflux, and the knockdown of SR-BI resulted in a slight increase in the efflux. These data are in accordance with a recent study that used liver-specific ABCA1-knockout mice to prove the role of ABCA1 in cholesterol efflux in hepatocytes (31, 32). In addition, extracellular apoA-I is another determinant for ABCA1-mediated cholesterol efflux (33). Because the apoA-I mRNA or secretion into the medium is not altered by MCP-1, we speculated that the reduced cholesterol efflux to apoA-I by MCP-1 resulted from suppression of ABCA1 expression in hepatocytes. The ABCA1 transporter resides on the cell surface and in intracellular compartments, and ABCA1 functions in lipid efflux and HDL biogenesis at the cell surface rather than in the intracellular compartments (16, 19). Our results showed a parallel suppression of both cholesterol effluxes to apoA-I and ABCA1 cell-surface localization by MCP-1. In addition, both of these effects can be corrected by PI3K activation, whereas the ABCA1 total protein was not regulated by PI3K activity, indicating that the cholesterol efflux was directly associated with ABCA1 cell-surface localization. Therefore, we suggest that the decreased cholesterol efflux by MCP-1 may be due to deficient ABCA1 trafficking to the cell surface at the posttranslational level, which results in cholesterol deposits in hepatocytes and the impaired lipiddation of apoA-I to form HDL.

Unexpectedly, the regulation of ABC transporters ABCA1 and ABCG1 by MCP-1 differed in HepG2 cells. The mechanism responsible for the downregulation of ABCA1 by MCP-1 involves both transcription with a corresponding decrease in ABCA1 mRNA levels and posttranslational mechanisms, whereas the ABCA1 total protein was not regulated by PI3K activity, indicating that the cholesterol efflux was directly associated with ABCA1 cell-surface localization. Therefore, we suggest that the decreased cholesterol efflux by MCP-1 may be due to deficient ABCA1 trafficking to the cell surface at the posttranslational level, which results in cholesterol deposits in hepatocytes and the impaired lipiddation of apoA-I to form HDL.

**Discussion.**

The differential regulation of ABC transporters ABCA1 and ABCG1 by MCP-1 differs in HepG2 cells. The mechanism responsible for the downregulation of ABCA1 by MCP-1 involves both transcription with a corresponding decrease in ABCA1 mRNA levels and posttranslational mechanisms, whereas the ABCA1 total protein was not regulated by PI3K activity, indicating that the cholesterol efflux was directly associated with ABCA1 cell-surface localization. Therefore, we suggest that the decreased cholesterol efflux by MCP-1 may be due to deficient ABCA1 trafficking to the cell surface at the posttranslational level, which results in cholesterol deposits in hepatocytes and the impaired lipiddation of apoA-I to form HDL.
phosphorylate the serine/threonine protein kinase Akt (36, 37). A key role of PI3K is its involvement in vesicular trafficking (38), including the recruitment of regulatory proteins such as the insulin-responsive glucose transporter-4 (39) to the plasma membrane. Our study illustrated that PI3K activity regulated the cell-surface protein expression, but not the total protein expression level, of ABCA1, ABCG1, and SR-BI in HepG2 cells. Meanwhile, p-Akt was repressed by MCP-1 in HepG2 cells that were pretreated with insulin, and PI3K activation could correct the MCP-1-induced decrease in the amounts of ABCA1, ABCG1, and SR-BI at the cell surface, which indicated that MCP-1 behaved like a PI3K inhibitor (such as wortmannin). However, another study (40) indicated that MCP-1 stimulates two separate PI3K isoforms, p85/p110 PI3-kinase and PI3K-C2a, in THP-1 cells. The variations in PI3K activity in response to MCP-1 may result from the existence of multiple isoforms of PI3K and the different cell types investigated (41). Moreover, there are different beliefs about whether the cellular effects of MCP-1 are mediated independently of the C-C chemokine receptor type 2 (42).

MCP-1 may potentially reduce reverse cholesterol transport in two ways and consequently increase the risk for atherosclerosis. On one hand, the cell-specific deletion of ABCA1 demonstrates that hepatocytes generate 70–80% of the plasma nascent HDL pool (43), which contributes significantly to the antiatherogenic process of RCT by regulating extracellular cellular cholesterol efflux. Because the cholesterol efflux to apoA-I is essential for generating nascent HDL (44, 45), which is the first step of RCT, the reduction of RCT by MCP-1 may be due to the impaired cholesterol efflux to apoA-I from hepatocytes, which is dependent on cell-surface expression of ABCA1. On the other hand, the reduction in RCT by MCP-1 may result from the impaired terminal step of RCT, namely, the reduced HDL-cholesterol lipid uptake by HepG2 cells for biliary secretion. Previous studies have demonstrated that the bulk of SR-BI-mediated lipid uptake occurs at the plasma membrane. The present study indicates that PI3K posttranslationally regulates the MCP-1-induced reduction of cell-surface expression of SR-BI and Dil-HDL lipid uptake but does not modify the total protein expression of SR-BI, which indicates the alternation in lipid uptake is mostly caused by the changes in the cell-surface expression of SR-BI. We suggest that MCP-1 impairs RCT activity in hepatocytes through the posttranslational regulation of ABCA1 and SR-BI cell-surface expression by PI3K/Akt, which may be an important mechanism that underlies the pro-atherogenic effects that are associated with inflammation.

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