Inhibition of ERK1/2 and Activation of Liver X Receptor Synergistically Induce Macrophage ABCA1 Expression and Cholesterol Efflux*

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ATP-binding cassette transporter A1 (ABCA1), a molecule mediating free cholesterol efflux from peripheral tissues to apoAI and high density lipoprotein (HDL), inhibits the formation of lipid-laden macrophage/foam cells and the development of atherosclerosis. ERK1/2 are important signaling molecules regulating cellular growth and differentiation. The ERK1/2 signaling pathway is implicated in cardiac development and hypertrophy. However, the role of ERK1/2 in the development of atherosclerosis, particularly in macrophage cholesterol homeostasis, is unknown. In this study, we investigated the effects of ERK1/2 activity on macrophage ABCA1 expression and cholesterol efflux. Compared with a minor effect by inhibition of other kinases, inhibition of ERK1/2 significantly increased macrophage cholesterol efflux to apoAI and HDL. In contrast, activation of ERK1/2 reduced macrophage cholesterol efflux and ABCA1 expression. The increased cholesterol efflux by ERK1/2 inhibitors was associated with the increased ABCA1 levels and the binding of apoAI to cells. The increased ABCA1 by ERK1/2 inhibitors was due to increased ABCA1 mRNA and protein stability. The induction of ABCA1 expression and cholesterol efflux by ERK1/2 inhibitors was concentration-dependent. The mechanism study indicated that activation of liver X receptor (LXR) had little effect on ERK1/2 expression and activation. ERK1/2 inhibitors had no effect on macrophage LXRA/β expression, whereas they did not influence the activation or the inhibition of the ABCA1 promoter by LXR or sterol regulatory element-binding protein (SREBP). However, inhibition of ERK1/2 and activation of LXR synergistically induced macrophage cholesterol efflux and ABCA1 expression. Our data suggest that ERK1/2 activity can play an important role in macrophage cholesterol trafficking.

Development of atherosclerotic lesions in coronary arteries is an underlying cause of coronary heart disease. Lipid-laden macrophage/foam cells are a prominent part of atherosclerotic lesions (1). Cellular cholesterol content in macrophages is determined by uptake and efflux of cholesterol (2). The type A scavenger receptor and type B scavenger receptor (CD36) mediate the binding and internalization of modified low density lipoprotein (LDL),3 thus, demonstrating pro-atherogenic properties (3, 4). In contrast, type B1 scavenger receptor and ATP-binding cassette transporter A1 (ABCA1) can mediate cellular free cholesterol efflux to extracellular high density lipoprotein (HDL) or lipid-free apolipoprotein A1 (apoAI) thereby inhibiting the development of atherosclerosis (5, 6). Compared with bi-directional cholesterol transport across cellular membranes that are mediated by type B1 scavenger receptor (7, 8), ABCA1 stimulates free cholesterol efflux from macrophages and other peripheral cell types to apoAI and/or HDL by using the energy from ATP hydrolysis (9). The binding of free cholesterol and phospholipids to apoAI also leads to the generation of nascent HDL (10). ABCA1 can also mediate cholesterol efflux to exogenous apolipoprotein E (apoE) (11). Although the removal of macrophage cholesterol by endogenous apoE is an ABCA1-independent process (12), ABCA1 activity can contribute to basal constitutive secretion of apoE from macrophages (13).

Anti-atherogenic properties of ABCA1 have been well investigated in both humans and animal models. In humans, mutations in ABCA1 expression cause Tangier disease, which is characterized by very low levels of serum HDL cholesterol, rapid catabolism of apoAI, severe cholesteryl ester accumulation in peripheral tissues, and a high risk of development of coronary heart disease (14–16). In animal models, overexpression of human ABCA1 reduces total cholesterol levels and atherosclerosis, whereas selective suppression of macrophage ABCA1 increases atherosclerosis without affecting total cholesterol levels (1, 6, 17, 18).

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3 The abbreviations used are: LDL, low density lipoprotein; ABCA1, ATP-binding cassette transporter A1; HDL, high density lipoprotein; apoAI, apolipoprotein AI; apoE, apolipoprotein E; LXR, liver X receptor; SREBP, sterol regulatory element-binding protein; LXRE, LXR response element; ERK1/2, extracellular signal-regulated kinases 1 and 2; MKP, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; EGF, epidermal growth factor; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; siRNA, small interference RNA; PKA, protein kinase A; JNK, c-Jun N-terminal kinase.
Expression of ABCA1 can be up-regulated by liver X receptor (LXR) and down-regulated by sterol regulatory element-binding proteins (SREBPs) 1,2 (19, 20). After ligand binding, LXR forms a heterodimer with another nuclear protein, retinoid X receptor. The heterodimer of LXR/retinoid X receptor binds to the LXR response element (LXRE) in the proximal region of ABCA1 gene promoter and induces ABCA1 transcription. Thus, synthetic LXR ligands can inhibit the development of atherosclerosis in animal models (21–23). In contrast, SREBP2 binds to the E-box in ABCA1 promoter to reduce ABCA1 expression (24). In macrophages, SREBP1 suppresses ABCA1 expression in an E-box-independent manner (19). Most of the oxysterols can suppress SREBPs. Therefore, they increase ABCA1 expression. Interestingly, some oxysterols, such as 22(R)-hydroxycholesterol, function as LXR ligands and SREBP suppressors simultaneously. The simultaneous activation of LXR and inactivation of SREBP by this type of oxysterols synergistically induce ABCA1 expression. In addition to transcription factors, the cellular ABCA1 levels are also regulated by post-translational mechanisms. ABCA1 is a molecule with a half-life of ~1–2 h. Thus, the decreased ABCA1 degradation by apoAI results in increased ABCA1 levels, whereas the enhanced ABCA1 degradation by unsaturated fatty acids decreases ABCA1 levels (25, 26).

Extracellular signal regulated kinases 1 and 2 (ERK1/2) or p44/42 mitogen-activated protein kinases (p44/42 MAPK) belong to a highly conserved family of Ser-Thr protein kinases and have been characterized to function through the Ras-Raf-MEK-ERK1/2 cascade (27). ERK1/2 are implicated in wide cellular processes, such as in embryogenesis, differentiation, proliferation, and cell death (28). ERK1/2 are ubiquitously expressed in all tissues/cell types and are strongly activated by multiple stimuli, including growth factors, such as epidermal growth factor (EGF) (28). In fact, overexpression or constitutive activation of ERK1/2 pathway can lead to progression of several cancers. Inhibitors of ERK1/2 have been investigated as potential therapeutic targets for cancer treatment (27). ERK1/2 have also been demonstrated to play a role in several aspects of cardiac system, such as cardiac development, hypertrophy, and protection (29). However, it is unclear if ERK1/2 play an important role in macrophage cholesterol metabolism and trafficking as well as the development of atherosclerosis. In this study, we investigated the effects of ERK1/2 activity on macrophage free cholesterol efflux and ABCA1 expression. We found that inhibition of ERK1/2 greatly increased macrophage free cholesterol efflux to apoAI and HDL. This increased cholesterol efflux was associated with ABCA1 expression. Further, the increased macrophage ABCA1 expression occurred by enhancing ABCA1 stability at both mRNA and protein levels. Although ERK1/2 inhibitor-induced ABCA1 expression was independent of LXR activation, ERK1/2 inhibitor, and LXR ligand synergistically induced macrophage cholesterol efflux and ABCA1 expression. Our studies reported herein describe a new function for ERK1/2 in cholesterol trafficking processes.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Inhibitors for different kinases were purchased from CalBiochemistry (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich except as indicated. Rabbit anti-ABCA1 polyclonal antibody was obtained from Novus Biologicals (Littleton, CO). Rabbit anti-total ERK1/2 and phospho-ERK1/2 polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-LXRα and LXRβ polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Acetylated low density lipoprotein, HDL, and apoAI were prepared as described (24, 30). The SDS-PAGE analysis indicated that the prepared HDL did not contain apoE.

**Cells**—RAW cells, a murine macrophage cell line, were purchased from ATCC (Rockville, MD) and cultured in complete RPMI medium containing 10% fetal calf serum, 50 μg/ml of penicillin/streptomycin, and 2 mM glutamine. At ~90% confluence, cells were switched to serum-free medium and received treatment.

To collect peritoneal macrophages, C57 wild-type mice (Jackson Laboratory, Bar Harbor, ME) were injected with 3 ml of 4% thioglycolate and maintained with access to water and normal chow for 5 days. Peritoneal macrophages were collected from the mouse abdomen by lavage with PBS. Cells were cultured in complete RPMI medium for 3 h, and all floating cells were removed. Adhesive cells were then cultured with complete RPMI medium for additional 2 days and then treated as indicated.

**Determination of Free Cholesterol Efflux from Macrophages**—Macrophages in 12-well plates were labeled in macrophase serum-free medium (Invitrogen, 1.5 ml/well) containing 50 μg/ml acetylated low density lipoprotein (used as the carrier for free cholesterol labeling) and 150 nCi/ml [3H]cholesterol for 24 h. After treatment, cells were washed twice with PBS and incubated for 1 h in serum-free medium, then switched to serum-free medium or medium containing purified apoAI (10 μg/ml) or HDL (15 μg/well). After 5-h incubation at 37 °C, medium from each well was collected for determination of radioactive activity in supernatants. The remaining cells were lysed by addition of 0.2 N NaOH, and the lysate was determined for protein content which was used to normalize cholesterol efflux (dpm/μg of protein).

**Northern Blot and Real-time Reverse Transcription-PCR Analysis of ABCA1 mRNA**—Total cellular RNA was extracted from cells in 60-mm dishes and used to determine expression of ABCA1 mRNA by Northern blot and quantitative real-time reverse transcription-PCR. The Northern blot was performed as described previously (31), and the probe for mouse ABCA1 mRNA was generated by reverse transcription-PCR with the following primers: forward, 5′-TGGACATCCTGAAGCCAG-3′, and backward, 5′-TTCTTCCACATGCCCT-3′.

To quantitative analyze ABCA1 transcript, 1 μg of total RNA was used to synthesize the first strand DNA with oligo(dT)18. The real-time PCR was performed by using SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and the following primers. Abca1: forward, 5′-CTCAGTGGCTAGCTGCTG-3′; backward, 5′-TACGCGCTACAGAGATCGG-3′; Gapdh: forward, 5′-ACAATTTGGCAATTGGGAA-3′; backward, 5′-GATGCAGGATGTTCTC-3′. The quantitative results for ABCA1 were normalized by the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA.
Regulation of ABCA1 Expression by ERK1/2

**Western Blot Analysis of ABCA1, LXRα, LXRβ, and Total and Phospho-ERK1/2**—Whole cellular proteins were extracted as follows: after treatment cells were washed twice with cold PBS, then scraped and lysed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 50 μg/ml aprotinin/leupeptin). Lysate was sonicated for 20 cycles, then microcentrifuged for 15 min at 4 °C. The supernatant was transferred to a new test tube and stored at −20 °C.

Nuclear proteins were extracted as described (32) with minor modifications. Briefly, cells were firstly suspended in cold buffer A (20 mM Heps, pH 7.9, 10 mM NaCl, 3 mM MgCl₂, 0.1% Nonidet P-40, 10% glycerol, 0.2 mM EDTA, 50 μg/ml aprotinin/leupeptin) and incubated on ice for 20 min followed by centrifugation for 5 min with a Microfuge (Beckman) at 3,000 rpm and 4 °C. The resultant pellet of nuclei was washed once with buffer A and then buffer B (20 mM Heps, pH 7.9, 20% glycerol, 0.2 mM EDTA, 50 μg/ml aprotinin/leupeptin). The nucleic pel-let was re-suspended in buffer C (20 mM Heps, pH 7.9, 0.4 mM NaCl, 20% glycerol, 0.2 mM EDTA, 50 μg/ml aprotinin/leupeptin) and incubated on ice for 1 h with vortex several times. The suspension was centrifuged for 30 min at 14,000 rpm and 4 °C. The supernatant was collected and stored at −20 °C.

After the content was determined by Lowry method, whole cellular or nuclear proteins were loaded and separated on a 7% (for determination of ABCA1) or 12% (for determination of the rest proteins) SDS-PAGE and then transferred onto nylon enhanced nitrocellulose membrane. The membrane was blocked with a solution of 0.1% Tween 20/PBS (PBS-T) containing 5% fat-free milk and incubated on ice for 1 h with vortex several times. The suspension was incubated for 3 min at 4 °C followed by washing for 3 × 10 min with PBS-T buffer. The blot was re-blocked with PBS-T containing 5% milk followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature. After washing 3 × 10 min with PBS-T, the membrane was incubated for 1 min in a mixture of equal volumes of Western blot chemiluminescence reagents 1 and 2 and then exposed to film before development.

**FACS Assay of Macrophage Surface ABCA1 Protein and the Binding of ApoAl to Cells**—After treatment, macrophages were scraped and washed twice with PBS containing 1% fetal calf serum. Approximately 1 × 10⁶ cells from each sample were blocked for 30 min at room temperature with PBS containing 5% goat serum. After washing with PBS, cells were incubated with rabbit anti-ABCA1 antibody (1:100) for 1 h at room temperature. Cells were then incubated with goat anti-rabbit fluorescein isothiocyanate-conjugated IgG (1:50) for 45 min at room temperature. After washing with PBS, cells were subjected to flow cytometric evaluation.

To determine the binding of apoAI to macrophages, purified apoAI was fluorescein-conjugated with a reactive succinimidyl macrophage free cholesterol efflux to apoAI. Pre-labeled RAW cells were treated with EGF at the indicated concentrations overnight followed by determination of free cholesterol efflux to apoAI (10 μg/ml). *, significantly different from control at p < 0.05 by Student’s t test (n = 4).

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**FIGURE 1. Regulation of macrophage free cholesterol efflux by ERK1/2 activity.** A and B, inhibition of ERK1/2 increases macrophage free cholesterol efflux to apoAI and HDL, respectively. RAW macrophages in 12-well plates were pre-labeled as described under “Experimental Procedures” and then received treatment overnight. Free cholesterol efflux was performed by incubating the treated cells in serum-free medium containing apoAI (10 μg/ml) (A) or HDL (15 μg/ml) (B) for 5 h at 37 °C. Radioactivity in medium was determined and normalized by cellular protein content. Treatments were as follows: ERK1/2 inhibitors, PD98059 (PD, 40 μM) and U0126 (2 μM); protein kinase C inhibitor, calphostin C (Cal, 1 μM); PKA inhibitor, myristoylated PKA inhibitor amide 14-22 (PKAI, 1 μM); p38 MAPK inhibitor, SB203580 (SB, 2 μM), and JNKs inhibitor, JNK inhibitor II (JNKI-2, 2 μM). LXR ligand, T0901317 (T0, 200 nm), was used as a positive control. # and *, significantly different from the corresponding controls at p < 0.05 by Student’s t test (n = 4). C, EGF decreases...
ester of carboxyl fluorescein by using a labeling kit from Princon Separations (Adelphia, NJ). After lift, cells were washed twice with PBS and then incubated with 10 μg/ml of labeled apoAI in serum-free medium for 2 h at 37 °C. After washed twice with PBS, cells were used to determine the binding of apoAI by FACS.

Preparation of Plasmid DNA and Determination of ABCA1 Promoter Activity—CDNA encoding nuclear form of mouse SREBP1a (N-terminal 1–460 amino acids) was generated by PCR using the clone purchased from Invitrogen (cDNA clone MGC:66503 IMAGE:6824948) as template DNA and following primers: forward, 5′-ACTCAAGATCTCGATGGACGAGCTGCCTTCG-3′; backward, 5′-CCGCGGTCACCTAGACCTGGCTATCCCATAAAGG-3′. After sequence was confirmed, the PCR product was digested with BglII and KpnI followed by subcloning into an expression vector, pEGFP-C2 (pEGFP-nSREBP1a).

Mouse ABCA1 promoters were constructed by PCR with mouse genomic DNA and following primers. For ABCA1 promoter A (from 179 to 227): forward, 5′-GCCTCGAGGTCGCCGGTTTAAAGGGCG-3′; backward, 5′-CTGCAGACTCCTCTTACCTGTTTTCCACTTTG-3′. For ABCA1 promoter B (from 113 to 227): forward, 5′-GCCTCGAGCAGGAGGCCGAACGGGGCG-3′, and backward was same as in promoter A. After sequence was confirmed, the PCR product was digested by Xhol and HindIII followed by ligation with pGL4 luciferase reporter vector (Promega, Madison, WI), transformed, and amplified. To analyze ABCA1 promoter activity, 95% confluent 293 cells in 24-well plates were transfected with DNA for ABCA1 promoter, nSREBP1a and Renilla (for internal normalization) by using Lipofectamine™ 2000 (Invitrogen). After 24 h of transfection plus treatment, cells were lysed, and cellular lysate was used to determine the activity of firefly and Renilla luciferases by using the Dual-Luciferase® Reporter Assay System from Promega.

Transfection of siRNA—The siRNA against mouse ERK1 and ERK2, and the scrambled siRNA were purchased from Santa Cruz Biotechnology. RAW cells (~80% confluence) in a 6-well plate were transfected with siRNA of ERK1 and ERK2 (an equal amount of each was mixed), and scrambled siRNA using TransIT-TKO transfection kit from Mirusbio (Madison, WI).
After 24-h transfection, total protein was extracted from cells, and we then assessed total ERK1/2 and ABCA1 protein levels by Western blot.

Data Analysis—All experiments were repeated at least three times, and representative results are presented. Data generated from the free cholesterol efflux, the activity of ABCA1 promoters, and the real-time PCR were analyzed by paired t test (n = 4).

RESULTS

Regulation of Macrophage Free Cholesterol Efflux by Activity of ERK1/2—To investigate if the inhibition of a kinase affects macrophage free cholesterol efflux, cells were pre-labeled with [3H]cholesterol and separately received treatment with inhibitors for various kinases. Cells were also treated with an LXR ligand, T0901317, as a positive control. After overnight treatment free cholesterol efflux from macrophages to apoAI in response to these reagents was determined (Fig. 1A). Inhibition of ERK1/2 by PD98059 (IC50 values were 4 and 50 nM for ERK1 and ERK2, respectively) induced free cholesterol efflux to apoAI at a comparable level as an LXR ligand (~4-fold). Another inhibitor of ERK1/2, U0126 (IC50 values were 72 and 58 nM for ERK1 and ERK2, respectively) also markedly increased macrophage free cholesterol efflux (~3-fold). In contrast, inhibition of other kinases, such as protein kinase C by calphostin C (1 μM, IC50 50 nM), protein kinase A (PKA) by myristoylated PKA inhibitor amide 14–22 (1 μM, IC50 50 nM), p38α, β, and δ MAPKs (p38 MAPK) by SB203580 (1 μM, IC50 34 nM), and c-Jun N-terminal kinases (JNKs) by JNK inhibitor II (2 μM; IC50 values were 40 and 90 nM for JNK-1/2 and JNK-3, respectively) did not influence macrophage free cholesterol efflux to apoAI. Therefore, enhancement of macrophage cholesterol efflux to apoAI appears to be ERK1/2 inhibition-selective.

In addition to apoAI, HDL also functions as an acceptor for ABCA1-mediated cholesterol efflux. To determine if the inhibition of ERK1/2 increases free cholesterol efflux to HDL, pre-labeled macrophages received the same treatment as in Fig. 1A overnight followed by assessment of macrophage free cho-

Regulation of ABCA1 Expression by ERK1/2

A.

| Ctrl | PD98059  | U0126 |
|------|----------|-------|
| 5    | 10       | 20    |
| 40   | 0.5      | 1.0   |
| 2.0  | 5.0      |       |

ABCA1 protein

B.

| scrambled | ERK1/2   | siRNA |
|-----------|----------|-------|
| 0         | 100      | 72    |
| 25        | 50       | 58    |
| 100       |          |       |

ERK1/2 protein

ABCA1 protein

C.

| Control  | PD98059 | U0126 |
|----------|---------|-------|
| Ctrl     | 5       | 10    |
|          | 20      | 40    |
|          | 0.5     | 1.0   |
|          | 2.0     | 5.0   |

Cholesterol efflux to apoAI (% of Control)

D.

| control  | PD98059 | U0126 |
|----------|---------|-------|
| Ctrl     | 5       | 10    |
|          | 20      | 40    |
|          | 0.5     | 1.0   |
|          | 2.0     | 5.0   |

Free cholesterol efflux (% of total labeled)
losterol efflux to HDL. The similar observations obtained for apoAI showed that inhibition of ERK1/2, but not of other kinases, increased macrophage cholesterol efflux to HDL (Fig. 1B).

To further determine the effects of ERK1/2 activity on macrophage cholesterol efflux, pre-labeled cells were treated with EGF to increase ERK1/2 activity followed by assessment of cholesterol efflux to apoAI (Fig. 1C). In contrast to the inhibition, the activation of ERK1/2 by EGF significantly suppressed the macrophage free cholesterol efflux. Taken together, results in these experiments (Fig. 1, A–C) suggest that ERK1/2 activity can affect macrophage cholesterol efflux processes.

Inhibition of ERK1/2 Increased Expression of Macrophage ABCA1 and the Binding of ApoAI to Cells—ABCA1 is an important molecule regulating cellular free cholesterol efflux to apoAI and HDL. To determine if ERK1/2 inhibitor-induced macrophage free cholesterol efflux is due to increased ABCA1 levels, cells were treated with different kinase inhibitors. Changes in ABCA1 mRNA in response to these reagents were assessed by quantitative real-time reverse transcription-PCR. Consistent with the effects on cholesterol efflux, ERK1/2 inhibitors (PD98059 and U0126) increased ABCA1 mRNA levels. In contrast, inhibition of PKA, protein kinase C, p38 MAPK, and JNKs had little effect on ABCA1 mRNA levels (Fig. 2A).

To study if the increased macrophage ABCA1 mRNA levels by ERK1/2 inhibitors can lead to an increase in cell surface ABCA1 protein, after treatment cells were determined for surface ABCA1 protein levels by FACS (Fig. 2B). Clearly, inhibition of ERK1/2 by PD98059 and U0126 elevated cell surface ABCA1 protein levels. However, inhibition of other kinases had no effect on cell surface ABCA1 protein levels. Associated with the changes in cell surface ABCA1 protein, the binding of apoAI to cells, which is the initial step in ABCA1-mediated free cholesterol efflux, was also selectively increased by ERK1/2 inhibitors, but not by inhibitors for other kinases (Fig. 2C). In contrast to inhibition of ERK1/2, activation of ERK1/2 by EGF reduced macrophage ABCA1 protein levels in a concentration-dependent manner suggesting the reduced macropage cholesterol efflux by EGF is, at least partially, attributed to the EGF-inhibited ABCA1 expression (Fig. 2D).

To study the physiological relevance of ERK1/2 inhibitors on macrophage ABCA1 expression and whether the induction of macrophage ABCA1 expression is dependent on the concentration of ERK1/2 inhibitors, we isolated peritoneal macrophages from wild-type mice and treated them with PD98059 and U0126. Results in Fig. 3A show that both PD98059 and U0126 increased peritoneal macrophage ABCA1 expression. The inductive effect of ERK1/2 inhibitor on ABCA1 expression is semi-concentration-dependent. The maximal induction values of primary macrophage ABCA1 expression by PD98059 and U0126 were 20 and 2 μM, respectively (Fig. 3A). To further confirm the effect of ERK1/2 inhibition on macrophage ABCA1 expression, cells were transfected with siRNA against ERK1 and ERK2. Results in Fig. 3B indicated that the reduced ERK1/2 protein expression by siRNA increased ABCA1 protein expression. In addition, the study with ERK1/2 inhibitor concentrations demonstrated that the increase in macrophage cholesterol efflux was concentration-dependent (Fig. 3C).

To study the dynamic effects of ERK1/2 inhibitors on cholesterol efflux, we studied efflux by incubating cells in medium containing apoAI for different time periods. After ERK1/2 inhibitor treatment, macrophage cholesterol efflux was increased over time, reaching zero-order kinetic at 5 h. However, at all time points, ERK1/2 inhibitors substantially increased macrophage free cholesterol efflux to apoAI (Fig. 3D).

Induction of Macrophage Free Cholesterol Efflux and ABCA1 Expression by ERK1/2 Inhibitors Is Independent of Activity of LXR or SREBP1a—ERK1/2 inhibitors act similarly as an LXR ligand to induce macrophage free cholesterol efflux and ABCA1 expression. To study the involved mechanisms, we initially tested the effects of an LXR ligand on macrophage ERK1/2 expression and phosphorylation. Both concentration and time course studies demonstrated that LXR ligand did not influence the total and phosphorylated ERK1/2 levels (Fig. 4A) suggesting that the induction of macrophage ABCA1 expression and free cholesterol efflux by the LXR ligand was unrelated to ERK1/2 expression or activation.

To study if the induction of macrophage ABCA1 expression by ERK1/2 inhibitors is through activation of LXR, we assessed the effects of ERK1/2 inhibitors on LXR expression. After treatment, nuclear proteins were extracted and used to determine LXRA and LXRβ protein levels by Western blot. Neither PD98059 nor U0126 changed the profiles of LXRA and LXRβ in the nuclei (Fig. 4B).

The LXRE (positions from −68 to −53) in the ABCA1 promoter can increase ABCA1 transcription, whereas the E-box (positions from −148 to −142) can inhibit ABCA1 transcription (19, 20). To determine if the induction of macrophage ABCA1 expression by ERK1/2 inhibitors can occur by influencing the activity of LXRE or E-box in the ABCA1 promoter, we transfected 293 cells with the DNA of ABCA1 promoter A (from −179 to +227; this promoter includes motifs of the LXRE and the E-box), then treated transfected cells with ERK1/2 inhibitors. In contrast to the effects on levels of ABCA1 mRNA and protein, U0126 had no effect on ABCA1 promoter A activity, whereas PD98059 inhibited it in a concentration-dependent manner (Fig. 4C, left panel). We further determined the effects of ERK1/2 inhibitors on the activity of the ABCA1

**FIGURE 3.** ERK1/2 inhibitors induce macrophage ABCA1 expression and free cholesterol efflux in a concentration- and time-dependent manner. A, peritoneal macrophages were isolated from thioglycollate-elicited wild-type mice and cultured in complete RPMI medium for 2 days. Cells were then treated with ERK1/2 inhibitors, PD98059 and U0126, at the indicated concentrations in serum-free medium overnight. Total cellular proteins were extracted and used to determine ABCA1 protein expression by Western blot as described under “Experimental Procedures.” B, RAW macrophages in a 6-well plate were transfected with scrambled siRNA or the mixed siRNA against ERK1 and ERK2 (equal amount of each) at the indicated concentrations for 24 h as described under “Experimental Procedures.” C, pre-labeled RAW macrophages were treated with PD98059 (40 μM) and U0126 (2 μM) overnight. After washing with PBS, cells were incubated in serum-free medium containing 10 μg/ml apoAI for indicated times, and the radioactivity in medium was determined. Free cholesterol efflux was calculated as the percentage of total labeled free cholesterol.
Regulation of ABCA1 Expression by ERK1/2

A. Ctrl T0 conc (nM) Time (hrs) Pi-ERK1/2 ERK1/2
50 100 200 1 2 4 6

B. Ctrl PD U0 (µM) LXRα protein LXRβ protein
10 40 1 5

C. promoter A (-179 to +227) promoter B (-113 to +227)

Relative activity of ABCA1 promoter (ABCAl/pGL-4.4R luc)

|          | PD98059 | U0126 |
|----------|---------|-------|
| PD98059  | 5       | 20    |
| U0126    | 40      | 0.5   |
|          | 1.0     | 5.0   |

D. vector (0.2 µg/well) promoter A (-179 to +227, 0.2 µg/well) promoter A + 200 nM T0 promoter A + nSREBP1α promoter A + nSREBP1α + 40 µM PD promoter A + nSREBP1α + 1 µM U0

Relative activity of ABCA1 promoter (ABCAl/pGL-4.4R luc)

|          | 0.4    | 0.4    |
| vector   | 0.4    | 0.4    |
| promoter A -179 to +227 | 0.4    | 0.4    |
| promoter A + 200 nM T0  | 0.1    | 0.1    |
| promoter A + nSREBP1α    | 0.2    | 0.2    |
| promoter A + nSREBP1α + 40 µM PD | 0.3    | 0.3    |
| promoter A + nSREBP1α + 1 µM U0 | 0.3    | 0.3    |

E. Ctrl Cyclo Cyclo + PD Cyclo + U0

|          | 2 | 4 | 6 | Time of treatment (h) |
| vector   | 100 | 14 | 8 | 3 |
| promoter A -179 to +227 | 32 | 18 | 12 |
| promoter A + 200 nM T0  | 40 | 12 | 17 |
| promoter A + nSREBP1α    | 58 | 74 | 71 |
| promoter A + nSREBP1α + 40 µM PD | 71 | 78 |
| promoter A + nSREBP1α + 1 µM U0 | 78 | 69 |

F. Ctrl Act D Act D+PD Act D+U0

|          | 2 | 4 | 6 | Time of treatment (h) |
| ABCA1 mRNA | 100 | 37 | 31 | 32 |
| GAPDH mRNA  | 74 | 71 | 58 | 71 |
| ABCA1/GAPDH (% of Ctrl) | 105 | 71 | 78 | 78 |
promoter B (from −113 to +227), which includes the LXRE but not the E-box. We observed that the effects of ERK1/2 inhibitors on the ABCA1 promoter B (Fig. 4C, right panel) were similar to that on the ABCA1 promoter A.

Previously, we reported that SREBP1 inhibited ABCA1 expression by decreasing ABCA1 promoter activity (24). To test if ERK1/2 inhibitor-induced ABCA1 expression occurs by blocking SREBP1 action, 293 cells were co-transfected with DNA of active SREBP1α (nuclear form) and ABCA1 promoter A followed by treatment with ERK1/2 inhibitors. Results in Fig. 4D demonstrate that nSREBP1α inhibited ABCA1 promoter activity, and this inhibition was not reversed by U0126 but enhanced by PD98059. Thus, the induction of macrophage ABCA1 expression by ERK1/2 inhibitors was also independent of SREBP1 activity.

Increased ABCA1 can occur by post-transcriptional modifications. To test if ERK1/2 inhibitors increase macrophage ABCA1 levels by increasing its stability, we treated cells with cycloheximide to arrest cellular protein synthesis in the absence or presence of ERK1/2 inhibitors. ABCA1 is a quickly degradable protein, thus, in the presence of cycloheximide, ABCA1 protein declined dramatically and was almost undetectable after 6-h treatment. In contrast, ERK1/2 inhibitors (PD98059 and U0126) reduced the decline at all time points of treatment suggesting ERK1/2 inhibitors are able to reduce the degradation of ABCA1 protein (Fig. 4E). Moreover, we observed that ABCA1 mRNA also degrades quickly (t1/2 < 2 h) and that this degradation was also reduced by ERK1/2 inhibitors (Fig. 4F).

ERK1/2 Inhibitor and LXR Ligand Have Synergistic Effects on Macrophage Free Cholesterol Efflux and ABCA1 Expression—Although ERK1/2 inhibitor and LXR ligand increase macrophage free cholesterol efflux and ABCA1 expression by different pathways, they still may interact. To test this hypothesis, we initially treated pre-labeled cells with different concentrations of T0901317 in the absence or presence of 20 μM PD98059 or 2 μM U0126 overnight. We then evaluated the effects of treatment on macrophage cholesterol efflux (Fig. 5A). T0901317 induced macrophage free cholesterol efflux in a concentration-dependent manner. Co-treatment of the LXR ligand at different concentrations with 20 μM PD98059 or 2 μM U0126 demonstrated a strong synergistic effect on macrophage free cholesterol efflux. In fact, 20 μM PD98059 alone increased cholesterol efflux almost 3-fold, whereas 1 nM T0901317 had little effect on cholesterol efflux. However, the combination of 20 μM PD98059 and 1 nM T0901317 increased cholesterol efflux 4-fold. T0901317 alone at 200 nM increased cholesterol efflux >3-fold, and its combination with 20 μM PD98059 increased cholesterol efflux 8-fold. The combination of T0901317 with U0126 also demonstrated the similar synergistic effects on macrophage cholesterol efflux. U0126 alone at 2 μM increased cholesterol efflux 2-fold, and its combination with 1 nM or 200 nM T0901317 increased cholesterol efflux nearly 3-fold or >6-fold (Fig. 5A).

We next determined if a synergistic effect existed by combining a fixed concentration of T0901317 (50 nM) with different concentrations of PD98059 or U0126 (Fig. 5B). T0901317 alone increased cholesterol efflux 4-fold. PD98059 alone at 5 or 40 μM increased cholesterol efflux by 3- and 4-fold, but the combination of 5 or 40 μM PD98059 with 50 nM T0901317 increased cholesterol efflux 6- or 10-fold. Similarly, the combination of U0126 at different concentrations with 50 nM T0901317 also increased cholesterol efflux in a synergistic manner. U0126 alone at 0.5 or 2 μM increased cholesterol efflux 2- to 3-fold, but its combination with 50 nM T0901317 increased cholesterol efflux 5- or 10-fold (Fig. 5B). Clearly, the combination of T0901317 with PD98059 or U0126 over a wide range of concentrations can induce macrophage cholesterol efflux.

To study if the co-treatment of LXR ligand and ERK1/2 inhibitor may induce macrophage ABCA1 expression in a synergistic manner, we treated cells with different concentrations of T0901317 in the absence or presence of 20 μM PD98059 or 2 μM U0126 (Fig. 5C) or reciprocally with different concentrations of PD98059 or U0126 in the absence or presence of 5 nM T0901317 (Fig. 5D). Results shown in Fig. 5C demonstrate that PD98059 or U0126 synergized with different concentrations of LXR ligand-induced macrophage ABCA1 expression. Interestingly, LXR ligand can enhance the increased macrophage ABCA1 expression induced by different concentrations of PD98059 or U0126 in a synergistic manner (Fig. 5D).

DISCUSSION

Anti-atherogenic properties of ABCA1 have been well investigated in both humans and animal models. In humans, the mutation in ABCA1 is the cause of Tangier disease and familial hypoalphalipoproteinemia. The impaired cholesterol efflux from peripheral tissues leads to the defect in generation of nascent HDL and the presence of macrophage/foam cells throughout the body (33). In addition, patients suffer from hepatosplenomegaly, peripheral neuropathy, and frequently

**FIGURE 4.** Mechanisms by which ERK1/2 inhibitors induce macrophage ABCA1 expression. A, LXR ligand has no effect on ERK1/2 expression and phosphorylation. Macrophages in serum-free medium were treated with T0901317 at the indicated concentrations for 2 h or with 200 nM T0901317 for indicated times. Levels of total and phosphorylated ERK1/2 (Pi-ERK1/2) were determined by Western blot. B, ERK1/2 inhibitors do not affect LXR expression. Macrophages were treated with ERK1/2 inhibitors, PD98059 and U0126, at the indicated concentrations overnight. Nuclear proteins were extracted and used to determine expression of LXRα and LXRβ proteins by Western blot as described under “Experimental Procedures.” C, the effects of ERK1/2 inhibitors on ABCA1 promoter activity. 293 cells in 24-well plates were transfected with ABCA1 promoter DNA (0.2 μg/well) and Renilla luciferase DNA as described under “Experimental Procedures” and received the indicated treatment overnight. Activity of firefly or Renilla luciferase in cellular lysate was determined by using the Dual-Luciferase Reporter Assay System (n = 4). D, the effects of ERK1/2 inhibitors on SREBP1α-inhibited ABCA1 promoter activity. 293 cells in 24-well plates were transfected with DNA for ABCA1 promoter and nSREBP1α at the indicated concentrations. Cells were then treated with ERK1/2 inhibitors at the indicated concentrations overnight. Activity of ABCA1 promoter was determined as described above. E, ERK1/2 inhibitors increase macrophage ABCA1 protein stability. Macrophages were treated with 5 μM cycloheximide (Cycle) or cycloheximide plus 40 μM PD98059 (PD) or 1 μM U0126 (U0) for the indicated times. Total cellular proteins were extracted and used to determine ABCA1 protein by Western blot as described under “Experimental Procedures.” F, ERK1/2 inhibitors increase macrophage ABCA1 mRNA stability. Macrophages were treated with 2 μM actinomycin D (Act D) or actinomycin D plus 40 μM PD98059 (PD) or 1 μM U0126 (U0) for the indicated times. Total cellular RNA was extracted and used to determine ABCA1 mRNA levels by Northern blot as described under “Experimental Procedures.” The same blot was hybridized with 32P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.
premature coronary heart disease (16, 33). In animal models, genetic deletion of ABCA1 results in the pathophysiologic conditions similar to those found in Tangier disease patients, such as reduced plasma cholesterol levels, which is mainly due to decreased HDL levels and the accumulation of lipid-laden macrophage/foam cells (34). In contrast, overexpression of ABCA1 leads to increased macrophage free cholesterol efflux, improved plasma lipid profiles, and reduced lesions in pro-atherogenic mice (6, 17, 35, 36). Interestingly, alteration of ABCA1 expression tissue specifically leads to different observations: 1) either overexpression or inactivation of hepatic ABCA1 can lead to the development of atherosclerosis (37, 38) and 2) activation of macrophage ABCA1 can inhibit atherosclerosis, whereas inactivation of macrophage ABCA1 can increase atherosclerosis (18, 37, 39).

Activation of LXR by specific ligands significantly induces macrophage ABCA1 expression and free cholesterol efflux. Synthetic LXR ligands have been demonstrated to inhibit or regress the development of atherosclerosis in animal models (21–23). However, activation of LXR can also induce expression of genes for fatty acid synthesis, such as SREBP1c, fatty acid synthase, stearoyl-CoA desaturase 1, and acyl-CoA carboxylase (40–42). In fact, administration of T0901317 to wild-type mice can increase hepatic triglycerides, plasma very low density lipoprotein-triglycerides, and secretion of very low density lipoprotein-triglycerides. It also induces hypertriglyceridemia in apoE−/− LDLR−/− double knockout mice, APOE*3-Leiden transgenic mice, and db/db diabetic mice (43, 44). Unfortunately, severe lipogenesis reduces the potential use of the synthetic LXR ligands for therapeutic treatment of atherosclerosis.

LXRα is expressed primarily in liver, intestine, adipose tissue, and macrophages, whereas LXRβ is constitutively expressed in many cell types (45). Genetic deletion of
ABCA1 increases ABCA1-mediated phospholipid efflux. However, ERK1/2 inhibitor synergizes with LXR ligand-induced ABCA1 expression and cholesterol efflux even when partial ERK1/2 activity is inhibited. Indeed, we observed that co-treatment of cells with an ERK1/2 inhibitor and an LXR ligand greatly increases macrophage cholesterol efflux to apoAI (Fig. 5). Others have found that reduced doses of T0901317 can inhibit atherosclerosis in LDLR−/− mice and is associated with lesser adverse effects on lipogenesis (23). Our findings imply that it may be feasible to use in vivo the combined ERK1/2 inhibitor and LXR ligand at very low doses to inhibit/ regress atherosclerosis without unfavorable lipogenic effects.

In summary, our results demonstrate that blockage of ERK1/2 increases macrophage ABCA1 expression and free cholesterol efflux to apoAI and HDL. ERK1/2 inhibitors increase ABCA1 expression at both mRNA and protein levels. In addition, ERK1/2 inhibitors synergize with LXR ligand-induced ABCA1 expression and cholesterol efflux. Taken together, our studies suggest a new function of ERK1/2 activity in cholesterol trafficking in the macrophage.

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