Glucocorticoid Receptor Regulates ATP-Binding Cassette Transporter-A1 Expression and Apolipoprotein-Mediated Cholesterol Efflux From Macrophages

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Objective—The ATP-binding cassette transporter-A1 (ABCA1) regulates cholesterol efflux from cells and is involved in high-density lipoprotein metabolism and atherogenesis. The objective of this study was to investigate the effect of dexamethasone (Dex) and other glucocorticoid receptor (GR) ligands on apolipoprotein AI–mediated cholesterol efflux from macrophages and ABCA1 expression in them.

Methods and Results—Dex, a GR agonist, decreased ABCA1 mRNA levels in a dose- and time-dependent fashion, and RU486, a GR antagonist, reversed the inhibitory effect of Dex. The effects of Dex and RU486 on ABCA1 protein levels and apolipoprotein AI–mediated cholesterol efflux from the macrophages were consistent with these changes in mRNA levels. Transfected RAW264.7, together with a human ABCA1 promoter–luciferase construct, inhibited transcriptional activity by Dex and overexpression of human GR. Transrepression by GR was not mediated by liver X receptor (LXR), because there were no differences in the effects of the GR ligands on promoter activity between a reporter construct with mutations at the LXR binding site and one without the mutations, and no changes were brought about in ABCG1 and ABCG4 expression by GR ligands.

Conclusions—Our results showed that GR ligands affected ABCA1 expression and cholesterol efflux from macrophages, which are regulated by GR through a LXR-independent mechanism. (Arterioscler Thromb Vasc Biol. 2006;26:163-168.)

Key Words: ABCA1 ■ dexamethasone ■ GR ■ cholesterol ■ macrophage

Multiple lines of evidence suggest that long-term use of glucocorticoids is associated with increased risk of developing atherosclerotic disease. It has been reported that glucocorticoid treatment can cause cardiovascular complications in patients with rheumatoid arthritis and systemic lupus erythematosus receiving such treatment and that patients with Cushing’s syndrome had a higher incidence of cardiovascular disease (CVD). In this regard, it has been noted that glucocorticoid treatment causes hypertension, as well as impaired glucose and lipid metabolism, which are classical risk factors for CVD. Other than this, glucocorticoids have been observed to directly affect the vascular wall with respect to cholesterol metabolism in the cells involved in atherogenesis.

Macrophage-derived foam cells containing accumulations of cholesteryl ester (CE) as lipid droplets are characteristically present in atherosclerotic plaque. In this connection, several investigators have reported that dexamethasone (Dex) induced CE accumulation in macrophages by enhancing scavenger receptor and acyl coenzyme A:cholesterol O-acyl-transferase activity and reducing cholesterol esterase activity.

Cholesterol efflux is a pivotal event in maintaining intracellular cholesterol levels and preventing the formation of macrophage-derived foam cells. Furthermore, the ATP-binding cassette transporter A1 (ABCA1) has been identified as a defective gene in Tangier disease, which is characterized by an extremely low level of circulating high-density lipoprotein (HDL), and accumulation of CE in various tissues, which is concentrated in macrophage-derived foam cells. In addition, ABCA1 has been shown recently to play an important role in apolipoprotein AI (apoAI)–mediated cholesterol efflux from peripheral cells and macrophages. Thus, given the key role of ABCA1 in facilitating cholesterol efflux, it would be of interest to determine how the ABCA1 gene itself is regulated. The expression of ABCA1 is greatly regulated by cAMP and sterols.

Oxysterols and 9-cis-retinoic acid (9cRA) bind liver X receptor (LXR) and retinoid X receptor (RXR), respectively, to form heterodimers, which bind to conserved consensus
cis-element, direct repeat 4 (DR4), in the ABCA1 promoter region, resulting in the activation of transcription.\textsuperscript{14,15} LXR, RXR, and the glucocorticoid receptor (GR) are all nuclear receptors that have been shown to play important physiological roles in macrophages.\textsuperscript{16} Recent studies have demonstrated that the peroxisome proliferator–activated receptor-\(\gamma\) upregulates LXR expression resulting in elevated ABCA1 levels in macrophages\textsuperscript{17} and that thyroid hormone receptor (TR) attenuated ABCA1 expression is dependent on LXR/RXR,\textsuperscript{18} but it is still unclear whether it is glucocorticoids or GRs that influence cholesterol efflux and ABCA1 expression in macrophages.

In the present study, we found that the GR agonist Dex decreased apoAI-mediated cholesterol efflux and ABCA1 expression in macrophages, whereas the GR antagonist RU486 increased them, with ABCA1 expression in macrophages being regulated via GR.

**Methods**

The Methods section can be found in an online supplement available at http://atvb.ahajournals.org.

**Results**

**Inhibitory Effect of GR Agonist Dex on ABCA1 Expression in THP-1 Macrophages and Restoration of Expression by GR Antagonist RU486**

ABCA1 mRNA levels in the THP-1 macrophages decreased in a dose-dependent manner 6 hours after treatment with Dex and were restored by concomitant treatment with RU486, actually showing an increase compared with basal levels (Figure 1A). When the macrophages were treated with acetyl LDL (AcLDL) and the LXR/RXR agonists, 22HC and 9cRA, respectively, ABCA1 mRNA levels dramatically increased compared with basal levels. In this experiment, Dex attenuated ABCA1 mRNA levels, and RU486 restored them in the presence of AcLDL and the LXR and RXR agonists (Figure 1B and 1C).

Figure 1D shows that treatment with RU486 increased ABCA1 mRNA levels in a dose-dependent manner in the presence of untreated FBS but did not increase these levels in the presence of dextran-coated charcoal FBS. These findings indicate that increased ABCA1 expression because of RU486 stems from the endogenous glucocorticoid effect. We also investigated the effect of another GR agonist, triamcinolone, noting the attenuation of ABCA1 mRNA levels and antagonization by RU486 (Figure 1E). We also examined the changes in ABCA1 mRNA levels in THP-1 macrophages with time after treatment with Dex, finding that ABCA1 mRNA levels were slightly elevated after 2 hours and were then clearly lower at 4 hours and thereafter (Figure 2A). In the presence of untreated FBS, RU486 had increased ABCA1 mRNA levels after 2 hours, and the increase was maintained up to 6 hours (Figure 2B). Levels had decreased at 16 hours after treatment with RU486.

**Effects of GR Ligands on ABCA1 Protein Levels and ApoAI-Mediated Cholesterol Efflux**

Figure 3A shows the ABCA1 protein levels after treatment with Dex or RU486 in the presence and absence of AcLDL. Dex decreased ABCA1 protein levels and RU486 restored them, even after stimulation of ABCA1 expression by AcLDL. We also examined the effects of the GR ligands on apoAI-mediated cholesterol efflux from THP-1 macrophages. Dex significantly reduced cholesterol efflux attributed to apoAI, and RU486 attenuated this inhibitory effect (Figure 3B), which was consistent with the changes in ABCA1 mRNA and protein levels.
GR Ligands Regulate ABCA1 in Macrophages

Figure 3. Effects of GR ligands on ABCA1 protein levels and apoAI-mediated cholesterol efflux from THP-1 macrophages. A, Sixteen hours after treatment with vehicle, 1 μmol/L of Dex, and RU486 in the presence or absence of 50 μg/mL of AcLDL, THP-1 macrophages were lysed and subjected to Western blot analysis as described in Methods. Upper bands are specific for ABCA1, and lower bands are nonspecific bands. B, Cholesterol efflux from macrophages mediated by apoAI. After cholesterol labeling, THP-1 macrophages were incubated with 1 μmol/L of the GR ligands or vehicle in the presence of 1 μg/mL of KW-3033 and 20 μg/mL of human apoAI for 16 hours. Cholesterol efflux was determined as described in Methods. For "A," the results for 4 samples are presented as the mean ± SE. *P < 0.05 vs control; † P < 0.05 vs Dex 1 μmol/L; ‡ P < 0.05 vs wild-type with 22HC. P values were calculated using Mann–Whitney U test.

Effects of GR Ligands on ABCA1 mRNA in HMDM and RAW264.7 Cells

We conducted experiments using human monocyte-derived macrophages (HMDM) and murine RAW264.7 cells to investigate whether Dex exerted inhibitory effects on ABCA1 and mRNA levels in other lines of macrophages. By means of quantitative real-time RT-PCR, Dex was shown to significantly reduce ABCA1 mRNA levels in HMDM, and they were restored by RU486 (Figure 1A, available online at http://atvb.ahajournals.org). In RAW 264.7 cells (Figure 1B), Dex also reduced ABCA1 mRNA levels, and they were restored by RU486.

Regulation of ABCA1 Promoter Activity by GR Ligands through GR

To investigate whether GR ligands regulate ABCA1 mRNA levels in macrophages at the transcriptional level, a human ABCA1 promoter luciferase reporter construct spanning −940 to +110 bp (ABCA1-Luc) was transfected into RAW264.7 cells, and luciferase assay was performed. Dex inhibited ABCA1 promoter activity in a dose-dependent fashion, and RU486 completely restored it as shown in Figure 4A. In the case of overexpression of GR, ABCA1 promoter activity increased in the absence of Dex but decreased in its presence in a dose-dependent manner (Figure 4B). We also observed that when macrophages were treated with actinomycin D, the GR ligands had no effect on ABCA1 mRNA levels or mRNA stability (data not shown). These data suggest that GR ligands regulate ABCA1 expression in macrophages at the transcriptional level through GR.

Alteration of ABCA1 Promoter Activity by GR Ligands Independent of LXR

To investigate whether the effects of GR ligands on ABCA1 promoter activity are associated with LXR- and RXR-dependent transcriptional regulation, we used a mutant reporter construct, DR4mut (Figure 4C). The LXR ligand 22HC failed to induce promoter activity for DR4mut, whereas it increased promoter activity in the case of the wild-type reporter construct ABCA1-Luc (Figure 4D). There were no differences in the changes in promoter activity because of GR ligands between ABCA1-Luc and DR4mut. ABCG1 and ABCG4 are reportedly involved in cholesterol efflux from macrophages19,20 and are also known to be LXR-responsive genes.21–23 Figure IIA and IIB (available online at http://atvb.ahajournals.org) show that GR ligands did not affect mRNA levels of ABCG1 and ABCG4 in THP-1 macrophages, although they had been increased by LXR and RXR ligands. The above findings clearly demonstrate that transcriptional regulation in ABCA1 by GR ligands is independent of LXR. We also studied the effect of GR ligands on mRNA levels for scavenger receptor class B, type 1, which is reportedly involved in HDL-mediated cholesterol efflux,24 but noted no change in levels (Figure IIC).
Discussion

We demonstrated that a GR agonist and antagonist, respectively, inhibited and stimulated ABCA1 expression in several lines of macrophages and apoAI-mediated cholesterol efflux from THP-1 macrophages, which were regulated through GR. Glucocorticoids have been reported to be associated with an increased risk of CVD presumably because of their unfavorable effects with respect to classical CVD risk factors, such as hypertension, impaired glucose, and lipid metabolism. Other than these effects, glucocorticoids have been reported to directly affect the vascular wall with respect to cholesterol metabolism in the cells involved in atherogenesis. Hirsch and Mazzone reported that Dex enhanced the degradation of AcLDL in macrophages through increased lipoprotein binding, which could mean enhanced scavenger receptor activity. Cheng et al reported that Dex increased cholesterol O-acetyltransferase activity and decreased neutral cholesterol esterase activity, resulting in greater accumulation of CE in macrophages. The homeostasis of intracellular cholesterol in peripheral cells is mainly maintained through its influx and efflux. Cholesterol efflux should be more important to macrophages than other types of cells, because macrophages express scavenger receptor class A, which is not downregulated by excess intracellular cholesterol. However, the effects of glucocorticoids on cholesterol efflux from macrophages have still to be investigated. Our study demonstrated the inhibitory effect of Dex on ABCA1 expression levels (Figures 1, 2, and 3A; Figure I) and apoAI-specific cholesterol efflux from macrophages (Figure 3B), which might be part of the reason for CE accumulation observed in previous studies. However, scavenger receptor class B, type 1 was not affected by the GR ligands, as mentioned above (Figure IIC). Petrichenko et al reported that Dex decreased cholesterol efflux mediated by HDL from vascular smooth muscle cells, and Stein et al observed that Dex decreased HDL-mediated cholesterol efflux from skeletal muscle in mice, suggesting that altered ABCA1 expression was involved.

It has been reported that glucocorticoid therapy increases serum HDL cholesterol levels. Because GR is expressed in most organs, we investigated whether GR ligands affected ABCA1 expression in other types of cells. GR ligands did not change ABCA1 mRNA levels in NIH 3T3 cells, and Dex brought about a slight increase in levels in both HepG2 and Caco-2 cells, which was reversed by RU486 (data not shown). These results indicate that ABCA1 expression is regulated by GR ligands in a cell-specific manner, and partly explain the reason for the elevation of HDL cholesterol levels by glucocorticoids. Given that the contribution of ABCA1 in macrophages to serum HDL cholesterol levels is minimal, as reported by Haghpassand et al, decreased ABCA1 expression in macrophages because of glucocorticoids is unlikely to affect HDL cholesterol levels.

GR agonists regulate gene expression in various ways, at the transcriptional, posttranscriptional, and posttranslational levels. The classical mode of gene regulation by glucocorticoids, which accounts for most cases of positive gene regulation, is known to be mediated by interaction of ligand-activated GR with positive control elements, which are present in single or multiple copies upstream of or within target genes. However, compared with positive regulation of gene transcription by GR, negative regulation seems to be more complicated as described below. Dex reportedly destabilizes the mRNA of some genes presumably by affecting RNA binding proteins, which associate with 3'-untranslated lesions to stabilize mRNA. Lasa et al reported that Dex caused sustained expression of mitogen-activated protein kinase phosphatase 1 and phosphatase-mediated inactivation of mitogen-activated protein kinase p38, resulting in destabilization of Cox-2 mRNA. Kaplan et al reported that a p38 inhibitor decreased ABCA1 expression in macrophages, and this inhibitory effect was not at the transcriptional level. In our study, the GR ligands did not affect ABCA1 mRNA levels or alter mRNA stability when macrophages were treated with actinomycin D (data not shown). Regarding transcriptional regulation, Dex inhibited ABCA1 promoter activity in a dose-dependent manner, and RU486 antagonized the inhibitory effects of Dex in this respect (Figure 4), which is consistent with the changes in mRNA levels observed. We also found that overexpression of GR decreased ABCA1 promoter activity in the presence of Dex. These findings indicate that GR ligands regulate ABCA1 expression in macrophages at the transcriptional level through GR.

We also observed a stimulatory effect of overexpression of GR in the absence of Dex (Figure 4B). Xiao and DeFranco reported that overexpression of unliganded and liganded GR, respectively, activated and inactivated heat shock factor, a transcription factor that transactivates heat shock protein genes, such as hsp90 and hsp70. They suggested that the mechanism for increased transcriptional activity was unliganded GR-induced depletion of hsp70, which inactivates heat shock factor, resulting in activation of heat shock factor. In our study, overexpression of unliganded GRs might have affected unknown protein-regulated events involving ABCA1 expression, as Xiao and DeFranco observed in the case of heat shock factor. Another possible mechanism is the reciprocal effects on transcriptional activity of liganded and unliganded receptors reported by Tagami et al. They reported that transcription of genes, which was inhibited by liganded TR, was reciprocally activated by unliganded TR with enhancement by overexpression of nuclear corepressors. Because Xiao and DeFranco clearly showed that unliganded GR was present not only in the cytosol but also in the nucleus, unliganded GR might stimulate ABCA1 promoter activity through the mechanism reported by Tagami et al. Because we did not fully clarify the mechanism for the inhibitory effect of GR on ABCA1 transcription, it remains unclear whether activated GR is directly associated with the transcriptional machinery involved in ABCA1 expression. Given the cell-specific regulation of ABCA1 expression by GR ligands, GR might affect pathways recruited by macrophage-specific proteins associated with ABCA1 expression.

Dex treatment caused the transient increase in ABCA1 mRNA levels from 1 to 2 hours after beginning the experiment, and the levels gradually decreased from 4 hours onwards (Figure 2A). We also observed a difference in the times of maximal changes in ABCA1 expression between Dex and RU486, which were at 6 hours and 2 hours after treatment, respectively. The dependence on endogenous glu-
corticoids (Figure 1D) indicates that RU486 exerts a competitive effect on ABCA1 expression through GR antagonization. However, it is possible that different mechanisms are involved in the respective effects of Dex and RU486 and that they are the reason for the early and late times of maximal changes in ABCA1 expression. As described above, both liganded and unliganded GR reportedly affected not only gene transcription through binding control elements of target genes but also protein–protein interaction and intracellular protein distribution. Therefore, several mechanisms could be involved in the changes in ABCA1 expression attributed to GR ligands. Additional study will be needed to clarify the mechanism for the transient increase in ABCA1 expression because of Dex.

There are 2 main mechanisms for the negative regulation of gene transcription by GR, 1 of them involving interference with DNA binding of upstream or general transcription factors and the other a repression mechanism, which is independent of DNA binding. Ligand-activated GRs have been reported to interact with activator protein-1 (AP-1)\textsuperscript{38} and nuclear factor κB (NF-κB)\textsuperscript{39,40} resulting in decreased transcription of AP-1 and NF-κB–responsive genes involved in inflammation. The ABCA1 promoter has been found to have consensus sequences for AP-1 and NF-κB.\textsuperscript{10,13} However, although GRs can affect ABCA1 promoter activity by interfering with AP-1 and NF-κB, Kaplan et al\textsuperscript{41} reported that lipopolysaccharides induced ABCA1 expression in macrophages without activating transcription. Thus, additional studies will be needed to ascertain whether the inhibitory effect of GR on ABCA1 transcription is mediated through AP-1 and NF-κB.

Accumulating evidence indicates that LXR regulates many genes involved in cholesterol homeostasis in cells, such as ABCA1, ABCG1, and ABCG4. Recently, several investigators have demonstrated interactions between LXR and other nuclear receptors. TR has been reported recently to inhibit ABCA1 promoter activity through competition between TR/RXR and LXR/RXR heterodimers.\textsuperscript{14} Small heterodimer partner, an unusual nuclear receptor, because it does not have the typical DNA binding domain, has been reported to act as a corepressor for several nuclear receptors, including LXR. Brendel et al\textsuperscript{42} reported that small heterodimer partner attenuated ABCA1 and ABCG1 promoter activities by interacting with LXR. Our study revealed that GR-mediated transcriptional inhibition in ABCA1 is independent of LXR (Figure 4). In addition, the expression of other LXR target genes, ABCG1 and ABCG4, were not affected by GR ligands (Figure IIA and IIB).

Other recent studies have reported transcriptional regulation for both ABCA1 and ABCG1, not only by LXR and RXR and cholesterol loading, but also by zinc finger protein 202.\textsuperscript{43} Moreover, Lorkowski et al\textsuperscript{44} have reported that macrophages from patients with Tangier disease showed a compensated increase in ABCG1 expression. Although such evidence led us to believe that these 2 genes share similar mechanisms of regulation, we observed differences in the mechanisms of regulation of ABCA1 and ABCG1, and other researchers have also found this to be the case. Therefore, these genes may have their own unique functions, like that of ABCA1 in apoptosis.\textsuperscript{44}

As other possible mechanisms for GR-mediated transpression of ABCA1, there is the stimulation of ABCA1 expression in macrophages by transforming growth factor (TGF) β,\textsuperscript{45} and inhibition of smad-dependent transactivation through the binding of smad3 by GRs.\textsuperscript{46} To determine whether GR-mediated transpression in ABCA1 is associated with the TGF-β signaling pathway, we investigated the effect of a neutral antibody against TGF-β on the changes in ABCA1 mRNA levels by GR agonists. The presence of the antibody, however, caused no changes in ABCA1 mRNA levels (data not shown).

Our study indicated that the attenuated cholesterol efflux and ABCA1 expression in macrophages could contribute to glucocorticoid-associated cardiovascular risk. Additional investigation of the mechanisms by which GR regulates ABCA1 expression would, therefore, provide important information for the study of compounds that facilitate cholesterol efflux.

References


Glucocorticoid Receptor Regulates ABCA1 Expression and Apolipoprotein-Mediated Cholesterol Efflux from Macrophages

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On-line Supplement

Materials and Methods

Materials

Dex, RU486, 22(R)-hydroxy cholesterol (22HC), 9cRA and triamcinolone were purchased from Sigma (St. Louis, MO). Phorbol 12-myristate 13-acetate (PMA) was purchased from Wako Pure Chemical (Tokyo, Japan). Human apoAI was purchased from Carbiochem (San Diego, CA). The ACAT inhibitor, KW-3033, was kindly donated by Kyowa Hakko and rabbit anti-human ABCA1 antiserum by Dr. S. Yokoyama of Nagoya City University.¹²

Cell Cultures

All cell culture media used in this study were phenol red-free and supplemented with 10% fetal bovine serum (FBS) and antibiotics and serum steroids were stripped from FBS using dextran-coated charcoal (DCC-FBS).³ Cells were cultured in a humidified atmosphere at 37°C with 5% CO₂. We used DCC-FBS in all experiments unless otherwise specified. THP-1 cells (RIKEN CELL BANK, Tokyo, Japan) were
maintained in RPMI 1640 (Sigma) containing 10% FBS. The differentiation of THP-1 monocytes into macrophages was induced by culturing them in a six-well plate in the presence of 200 nM of PMA for 72 h, at a density of $2.0 \times 10^6$ cells/well. The macrophages so produced were equilibrated by incubation with RPMI 1640 containing 10% DCC-FBS for 24 h, and then used for the experiments. RAW264.7 cells were obtained from RIKEN CELL BANK (Tokyo, Japan) and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% DCC-FBS. Human peripheral blood monocytes were isolated using the method of Fogelman et al., with Ficoll/Hypaque gradient centrifugation. The mononuclear cells thus obtained were re-suspended in RPMI 1640 (Sigma) supplemented with 20% autologous serum, plated on to serum-treated 10-cm dishes and incubated for 2 h. Non-adherent cells were removed by washing three times with phosphate buffered saline (PBS), and adherent cells were then detached by incubation in PBS containing 5% autologous serum and 0.02% EDTA at 4°C for 30 min. The adherent cells were then washed extensively and re-suspended in RPMI 1640 supplemented with 5% autologous serum. They were then plated on 10-cm dishes and incubated for 10 days so that they would differentiate into macrophages.

**Northern blot analysis**

At the indicated hours after treatment with the GR ligands, Dex and RU486, and other compounds, total RNA was extracted from the cells using TRIzol (Invitrogen, Carlsbad, CA). Fifteen µg of total RNA was separated by electrophoresis using a 1% (w/v) agarose/formaldehyde gel and transferred to nylon membranes. The membranes were hybridized with the probes, which had been radio-labeled with $[\alpha-^{32}P]dCTP$, using a Random Prime DNA Labeling Kit (Takara Bio, Otsu, Japan).
Probes for human ABCA1, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mouse ABCA1, mouse GAPDH, human scavenger receptor class B, type I (SR-BI) (Gene Bank accession number, NM_005502, NM_002046, NM_013454, NM_008084, NM_005505, respectively) were constructed from cDNA fragments amplified by reverse transcription-polymerase chain reaction (RT-PCR) using cDNA obtained from THP-1 macrophages and RAW264.7 cells as a template. To quantify the relative expression in Northern blot analyses, densitometric measurement was performed using NIH image software.

**Immunoblotting of ABCA1**

Cells were harvested and suspended in 5 mM Tris-HCl buffer (pH 8.5) containing 1% protease inhibitor cocktails (Roche, Indianapolis, IN) and placed on ice for 30 min with occasional mixing by a vortex. Next, cell suspensions were centrifuged at 800 x g for 5 min, and then the supernatant was centrifuged at 100,000 x g for 30 min. The total membrane precipitated fraction was suspended in the same buffer. After determination of protein content using a DC-protein kit (Biorad, Hercules, CA), the membrane fractions were stored at -80 °C until use. The total membrane protein (30 µg) was dissolved in 0.9 M urea, 0.2% (v/v) Triton X-100, and 0.1% (w/v) dithiothreitol supplemented with 10% (w/v) lithium dodecylsulfate and then analyzed by electrophoresis using a 6% (w/v) polyacrylamide gel containing 0.1% (w/v) sodium dodecylsulfate followed by blotting onto a polyvinylidene difluoride membrane. The membrane was blocked in 5% skim milk and incubated with rabbit anti-human ABCA1 antisera for 1 h. After washing three times with PBS containing 0.05% Tween 20 (pH 7.5), the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham Bioscience) for 1 h. ABCA1 was visualized by a
chemiluminescence method (ECL Plus Western Blotting Detection System, Amersham Biosciences, Foster City, CA)

**Cholesterol loading and determination of cholesterol efflux**

Cholesterol efflux experiments were performed as described by Smith et al.\(^5\)

Low-density lipoprotein (LDL) was separated by sequential ultracentrifugation and acetyl LDL (AcLDL) was prepared according to the methods of Basu et al.\(^6\) After 72 h of THP-1 monocyte differentiation into macrophages, the macrophages so produced were cholesterol loaded and labeled in 0.5 ml of DGGB (DMEM supplemented with 50 mM glucose, 2 mM glutamine, and 0.2% BSA) containing 50 µg/ml of \(^3\)Hcholesterol-AcLDL and 1 µg/ml of KW-3033. On the following day, macrophages were washed twice with 0.2% BSA-PBS, and equilibrated with DGGB for 2 h. The medium was then replaced with DGGB containing the GR ligands, 1 µg/ml of KW-3033 and 20 µg/mL of human apoAI. After 16 h of incubation, the culture was centrifuged to remove cell debris and 100 µl of the medium was removed for determination of radioactivity. At the end of the chase period, the macrophages were dissolved in 0.5 ml of 0.2 M sodium hydroxide, and the radioactivity per aliquot was measured. The percentage cholesterol efflux was calculated by dividing the media-derived radioactivity by the sum of the radioactivity in the media and the macrophages.

**Real-time quantitative RT-PCR**

First-strand cDNA was synthesized from the total RNA (250 ng) by placing in a Reverse Transcription Reagent (Applied Biosystems). Quantitative PCR was performed using a Perkin–Elmer 7700 PCR machine, TaqMan PCR master mix and commercially available primers, and FAM-labeled TaqMan probes (Assays-on-Demand,
Expression data were normalized for GAPDH levels.

**Construction of Luciferase Reporter Plasmids**

The human ABCA1 promoter region spanning -940 to +110 bp was PCR-amplified using ABCA1-specific primers with HindIII sites (forward, 5'-CCCAAGCTTAAGTTGGAGGTCTGGAGTGG-3'; reverse, 5'-CCCAAGCTTACCGGCTCTGTTGGTGCGCGG-3') and a plasmid containing the nucleotides –2245 to +110 of the human ABCA1 promoter region derived from the human BAC clone as a template (kindly provided by Dr. S. Santamarina-Fojo of the National Institute of Health). The PCR-amplified product was ligated into a pGL3 Enhancer vector (Promega, Madison, WI) and confirmed by sequencing to create the ABCA1-Luc construct. To obtain a reporter construct with mutations in 5' half-site of ABCA1 DR4 element (designated as DR4mut), we performed site-directed mutagenesis using a Quick Change II Site-Directed Mutagenesis Kit (Stratagene La Jolla, CA) and the respective primers – forward: 5'-CGAGCGCAGAGGTTACTATCTGCAGAAGCCTGTGCTCTCCC -3' – reverse: 5'-GGGAGAGCACAGGCTTCTGCAGATAGTAACCTCTGCGCTCG -3'. The plasmid for expression of human GRα, pCMX-hGRα was kindly provided by Dr. R. M. Evans of the Salk Institute.

**DNA Transfection and Luciferase Assay**

Transfection was performed in the RAW 264.7 cells with FuGENE6 (Roche) in 24-well plates according to the manufacturer's instructions. Transfection was also performed with 190 µg of ABCA1-Luc, 10 µg of phRL-TK (Promega) and indicated doses of pCMX-hGRα. The pCMX empty vector was transfected so that total
transfected pCMX vectors were 100 ng/well. Eight hours after transfection, treatment with the GR ligands was initiated, and terminated at 30 h from the beginning of transfection. Cells were then lysed by the addition of 250 µL passive lysis buffer (Promega). Next, luciferase activity assays were performed using a Dual Luciferase Reporter Assay System (Promega) with activity read using a LB9501 luminometer (Berthold). The phRL-TK luciferase activity was used to correct for transfection inefficiency and other factors that could affect the results.

**Figure legends**

**Supplemental Figure I.** Effects of GR ligands on ABCA1 mRNA levels in various lines of macrophages. (A) HMDM were treated with 1 µM of the GR ligands or vehicle for 6 h. Total RNA was extracted and real-time quantitative RT-PCR was performed as described in Materials and Methods to determine ABCA1 mRNA expression levels. The relative ABCA1 mRNA abundance was calculated by dividing the values for the expression levels of ABCA1 by those for GAPDH. (B) Northern blot analysis. RAW264.7 cells were treated with 1 µM of the GR ligands or vehicle for 6 h. For A, the results for 4 samples are presented as the mean ± SE. * P<0.05 versus control; † P<0.05 versus Dex 1 µM. P values were calculated using Mann-Whitney's U test.

**Supplemental Figure II.** Effects of GR and LXR/RXR ligands on ABCG1 (A), ABCG4 (B) and SR-BI (C) mRNA levels in THP-1 macrophages. After equilibration with the media containing DCC-FBS, THP-1 macrophages were treated with the indicated doses of Dex (A), 1 µM of Dex (B, C), 1 µM of RU486, 10 µM of 22HC and 100 nM of 9cRA, or left untreated, for 6 h. Total RNA was extracted and Northern
blot analysis was performed for ABCA1 and SR-BI as described in Materials and Methods. Real-time quantitative RT-PCR was performed as described in Materials and Methods to determine ABCG4 mRNA levels. The relative ABCG4 mRNA abundance was calculated by dividing the values for expression levels of ABCA1 by those for GAPDH. For B, the results for 9 samples are presented as the mean ± SE. * P<0.05 versus control. P values were calculated using Mann-Whitney's U test.
References


**A**

![Bar chart showing fold change](chart)

**Relative mRNA Abundance (x10^-3)**

- 6.1 ± 0.3
- 3.4 ± 0.1
- 5.3 ± 0.3

**Treatment**

- Dex: - + +
- RU486: - - +

**B**

![Western blot images](blot)

**ABCA1**

- Dex: - + +
- RU486: - - +

**GAPDH**

- Dex: - + +
- RU486: - - +
A

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Dex (M) | $10^{-8}$ | $10^{-7}$ | $10^{-6}$ | $10^{-6}$ |
| RU486  | -         | -         | -         | -         |
| 22HC/9cRA| -         | -         | -         | -         |

B

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Relative mRNA Abundance ($\times10^6$) | 2.5±0.3 | 2.8±0.3 | 2.1±0.3 | 5.4±1.2 |
Dex                                  | -       | +       | +       | -       |
RU486                                | -       | -       | +       | -       |
22HC/9cRA                            | -       | -       | -       | +       |
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C

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Dex    | +     | +     |
RU486  | -     | +     |