# Lipid efflux in human and mouse macrophagic cells: evidence for differential regulation of phospholipid and cholesterol efflux

**Robert S. Kiss, Jovana Maric, and Yves L. Marcel**[1]

Lipoprotein and Atherosclerosis Research Group, University of Ottawa Heart Institute, and Departments of Pathology and Laboratory Medicine, and Biochemistry, Microbiology, and Immunology, Faculty of Medicine, University of Ottawa Heart Institute, Ottawa, Ontario, K1Y 4W7, Canada

**Abstract** **ABCA1 is a critical regulator of lipid efflux from cells, which is highly regulated at the transcriptional and posttranslational levels. However, cells from different species and different tissues, and primary versus immortalized cells, show different modes of regulation. We have carried out a comparative analysis of basic signaling pathways of lipid efflux in mouse J774 cells, mouse peritoneal macrophages (MPMs), human THP-1 cells, and human monocyte-derived macrophages. Cyclic AMP (cAMP) was a potent stimulator of lipid efflux in mouse macrophages, but not in human macrophages. Moreover, this cAMP-inducible component of efflux from MPMs was inhibitable by H89 [a protein kinase A (PKA) inhibitor], but H89 did not affect basal efflux. On the other hand, cAMP failed to show any stimulatory effect in human macrophages, but basal efflux was inhibitable by H89. In MPMs and THP-1 cells, protein kinase C (PKC) inhibitors blocked cholesterol efflux but had no effect on phospholipid efflux, demonstrating the separation of the regulation of phospholipid efflux and cholesterol efflux in macrophages.** We conclude that: *1)* **cAMP regulates lipid efflux predominantly in a PKA-dependent fashion;** *2)* **cholesterol efflux is modulated by a PKC-dependent mechanism; and** *3)* **mouse and human macrophages exhibit different modes of regulation of lipid efflux.**—Kiss, R. S., J. Maric, and Y. L. Marcel. **Lipid efflux in human and mouse macrophagic cells: evidence for differential regulation of phospholipid and cholesterol efflux.** ***J. Lipid Res.*** **2005.** 46: **1877–1887.**

**Supplementary key words** cholesterol efflux • macrophage • ATP binding cassette transporter A1 • lipid efflux • regulation

With the recent discovery of the role of ABCA1 in lipid efflux, and the potential regulation of ABCA1 as a means to regulate plasma HDL levels, much research has focused on the transcriptional and posttranslational regulation of ABCA1. There are multiple elements in the proximal promoter of ABCA1 that may regulate its expression (1). Cholesterol loading strongly stimulates ABCA1 expression in macrophages, where it evidently increases ABCA1 mRNA abundance and protein levels, although the mechanism is not understood (2). ABCA1 gene regulation is also mediated by the nuclear liver X receptors (LXR α and β) and retinoid X receptors (RXRs) (3, 4), and their respective ligands, oxysterols and 9-*cis*-retinoic acid (9cRA). Cavelier et al. (5) showed, in mice carrying a human ABCA1 transgene under the control of its natural flanking sequences, that the human and mouse ABCA1 genes responded differently to cyclic AMP (cAMP), and that novel, as-yet-unreported promoters may exist that regulate ABCA1 expression and activity. As such, cAMP significantly stimulates efflux in J774, mouse peritoneal macrophages (MPMs), and RAW 264 cells (6–10) and not in THP-1 cells or human monocyte-derived macrophages (MDMs) (11, 12). cAMP's primary effect may be on transcription and protein expression, because efflux is not stimulated before 6 h, and protein synthesis inhibitors prevent stimulation (13). At the other end, efflux was reduced to near baseline levels within 6 h upon removal of cAMP, indicating that ABCA1 is highly regulated at the protein level (8, 14–16). ABCA1 can be phosphorylated in vitro (17, 18), and this can be regulated by protein kinase A (PKA) (18, 19). Activators of PKA, including cAMP, are known to stimulate specific efflux and ABCA1 phosphorylation, whereas inhibitors of PKA cause decreased efflux and reduced phosphorylation (18, 20–23). Therefore, cAMP promotes lipid efflux by both stimulating expression of ABCA1 and activation of ABCA1 by phosphorylation.

It is known that many other signaling pathways can also affect lipid efflux. Janus kinase 2 appears to selectively modulate apolipoprotein interactions with ABCA1 without di-

*Manuscript received 8 December 2004 and in revised form 4 May 2005 and in re-revised form 23 June 2005.*

*Published, JLR Papers in Press, July 1, 2005.*
*DOI 10.1194/jlr.M400482-JLR200*

[1] To whom correspondence should be addressed.
e-mail: ylmarcel@ottawaheart.ca

rectly phosphorylating ABCA1 (24). Phosphorylation of a PEST sequence in ABCA1 promotes calpain-mediated degradation of ABCA1 (25). Protein kinase C (PKC) stimulators promoted cholesterol efflux in a dose-dependent fashion in normal and Tangier disease [(TD) ABCA1-defective] fibroblasts (26, 27), and deficient PKC activation was detected in TD fibroblasts (28). It was proposed that apolipoprotein A-I (apoA-I)-containing lipoproteins stimulate removal of sphingomyelin, which, in turn, stimulates phosphatidylcholine-specific phospholipase C (PC-PLC) and phospholipase D (PC-PLD) (29). The generation of diacylglycerol (DAG) then stimulates PKC, which phosphorylates ABCA1 (26, 29). PC-PLC and PC-PLD activity was reduced in TD fibroblasts (30), and treatment with pertussis toxin [(PTX) an inhibitory G-protein (Gi) inhibitor] could promote a TD-like activity of PLC and PLD in normal fibroblasts (30). In rat vascular smooth muscle cells (SMCs) and MPMs, PKC stimulators enhanced cholesterol efflux, whereas inhibitors reduced cholesterol efflux (31, 32). These authors showed that PKC effectors had no effect on phospholipid efflux in macrophages, SMCs, and some fibroblasts (32, 33) but had a small but significant effect on phospholipid efflux in other fibroblasts (29), suggesting an independent regulation of phospholipid and cholesterol efflux in some cells. Vanadate inhibited cholesterol efflux, but not phospholipid efflux, in SMCs (34) but not HEK 293 cells (35), also indicating that phospholipid and cholesterol efflux are independently regulated in some cells. On the other hand, Smith et al. found no evidence for independent regulation in RAW 267.4 and HEK 293 cells of phospholipid and cholesterol efflux, and found that treatment with high doses of cyclodextrin or vanadate induced cell death that might explain the perceived uncoupling of lipid efflux (36). Various cells, including fibroblasts, SMCs, macrophages, adipose cells, epithelial cells, endothelial cells, and hepatocytes, have all been examined for various properties of apoA-I-mediated efflux (7, 20, 37–42), but macrophagic cells are most severely affected by TD and are regulated differently than other cells.

In an attempt to reconcile numerous independent observations about lipid efflux regulation, we have evaluated apoA-I-specific efflux and its regulation in murine versus human macrophages and primary versus transformed macrophages, using mouse MPMs and J774 cells, and human MDMs and THP-1 cells.

## EXPERIMENTAL PROCEDURES

### Materials

[$^3$H]cholesterol was obtained from Perkin-Elmer. ApoA-I was obtained from a bacterial expression system as described previously (43). Dulbecco's modified Eagle's medium (DMEM) and RPMI were obtained from Gibco Invitrogen. 8-(4-Chlorophenyl thio) adenosine 3′,5′-cyclic monophosphate (CPT-cAMP), fetal bovine serum (FBS), BSA, 9cRA, phorbol 12-myristate 13-acetate (PMA), sodium vanadate, and Histopaque 1077 were obtained from Sigma. *N*-[2-(4-bromocinnamyl) aminoethyl]5-isoquinoline-sulfonamide (H89), PTX, cholera toxin (CTX), mastoparan, 1,2-dioctanoylglycerol (DOG) and calphostin C (CalC) were obtained from Calbiochem. Penicillin/streptomycin (P/S) was obtained from Gibco.

### Cell isolation and treatment

All procedures were approved by the University of Ottawa Heart Institute Human Ethics Research Board. Human MDMs were prepared following the procedure of Cohen et al. (44). The cells were plated at $4.4 \times 10^6$ cells/ml in 24-well cell culture plates. The media was replaced after 2 h, to wash away nonadherent cells. The cells were fed every 48 h and were maintained in culture for 12 days to allow the monocytes to differentiate into macrophages. Macrophage morphology, uptake of acetylated LDL (AcLDL) by class A scavenger receptors, and expression of CD68 were used to determine complete differentiation of MDMs.

MPMs were obtained by peritoneal lavage from Swiss-Webster mice (Charles River). Mouse J774 cells and MPMs were cultured in DMEM supplemented with 10% FBS and 1% P/S. Human THP-1 cells were grown in suspension in RPMI media supplemented with 10% FBS, 1% P/S, 1% L-glutamine, and 5 mM β-mercaptoethanol. They were differentiated by treatment with PMA (10 nM) for 72 h prior to the experiment.

### Isolation of LDL

LDL was obtained between the density limits 1.006 and 1.063 g/ml by sequential ultracentrifugation. AcLDL was prepared by the method of Goldstein et al. (45). Protein concentration of lipoproteins was calculated using the Markwell Lowry assay (46).

### Efflux assay

The cells were labeled with [$^3$H]cholesterol (5 μCi/ml) or [$^3$H]choline (5 μCi/ml) incorporated into AcLDL (37.5 μg AcLDL/ml) and incubated for 36 h. Cells underwent an equilibration step in media plus 2 mg/ml BSA for 12 h prior to efflux. After the equilibration period, cells were incubated with apoA-I (50 μg/ml) in DMEM/BSA (or RPMI/BSA) for 5 h. [$^3$H]cholesterol efflux was expressed as medium [$^3$H]cholesterol radioactivity as a percentage of total [$^3$H]cholesterol radioactivity (cells plus medium). [$^3$H]phospholipid efflux, and in some experiments [$^3$H]cholesterol efflux, was detected by immunoprecipitation of apoA-I and its associated lipids (42), and the results are expressed as total radioactivity immunoprecipitated/mg cell protein. Each condition was performed in the absence of apoA-I, and this efflux value was subtracted from the apoA-I efflux value, to obtain the apoA-I-specific efflux. Efflux from the sample containing only apoA-I (no treatment) was regarded as 100%. Each experiment was performed in triplicate. Results presented are the mean (±SD) of at least three independent experiments. Alternatively, prior to efflux, cell lipids were extracted with hexane (1 h, room temperature), the samples were loaded onto a Silica Gel 60 thin-layer chromatography plate, and the lipids were separated in the solvent system hexane-diethyl ether-acetic acid (105:45:1.5; v/v/v). Bands corresponding to free cholesterol (FC) and cholesteryl ester (CE) were scraped off the plate, and the radioactivity was determined by scintillation counting.

### Western blotting

Cells were grown under the conditions described up to the point prior to efflux, before being placed on ice. Cells were removed from the plate by scraping, were homogenized with 20 strokes in a Dounce homogenizer, and then were centrifuged at 2,000 *g* for 10 min at 4°C to remove unbroken cells and debris. The supernatant was electrophoresed on a 4% SDS-polyacrylamide gel, and transferred to nitrocellulose at 125 V for 4 h. An ABCA1 antibody (Novus Biologicals; 1:500 dilution) was used for probing, with an anti-rabbit secondary antibody conjugated with horseradish peroxidase (Amersham Biosciences) for detection.

## RESULTS

### Lipid efflux from macrophages under baseline conditions

ApoA-I-specific efflux from J774 cells, MPMs, THP-1 cells, and human MDMs was measured in cells labeled with [$^3$H] cholesterol delivered via AcLDL uptake and in the absence of any exogenous agents. J774 macrophages had the most robust efflux in absolute terms (percent of cell label), followed by MDM, MPM, and THP-1 (data not shown). When cholesterol efflux was measured (cpm as a function of total cell protein), the amount of efflux roughly correlated with the ratio of labeled CE to FC (CE/FC ratio; **Fig. 1**), demonstrating that, in general, the CE/FC ratio predicts the amount of cholesterol efflux. Importantly, there is an inverse correlation between efflux versus FC/total cholesterol ratio (data not shown), suggesting that the amount of CE is important for efflux.

### Independent effect of cAMP on ABCA1-mediated efflux

9cRA, an RXR ligand and enhancer of ABCA1 gene expression, cAMP, a stimulator of efflux, and CTX, a stimulatory G-protein enhancer, were used to stimulate efflux. We have completed time courses and concentration curves with each drug in each cell type in separate experiments and determined the best working conditions for each agent. Pretreatment with cAMP (150 μM), CTX (2 μg/ml) and 9cRA (10 μM) for 12 h proved to be most effective, and all subsequent experiments were performed under these conditions, unless noted. Identical treatment conditions and concentrations were used for each cell type to ensure comparability. In J774 cells, cAMP stimulated cholesterol efflux to 350% of control untreated cells (**Fig. 2A**). CTX increased cholesterol efflux to a level equivalent to the cAMP effect. 9cRA stimulated slightly more than cAMP. Although incubation of CTX and cAMP had no additive effect, incubation of 9cRA together with cAMP resulted in an additive effect, indicating that these agents have separate targets.

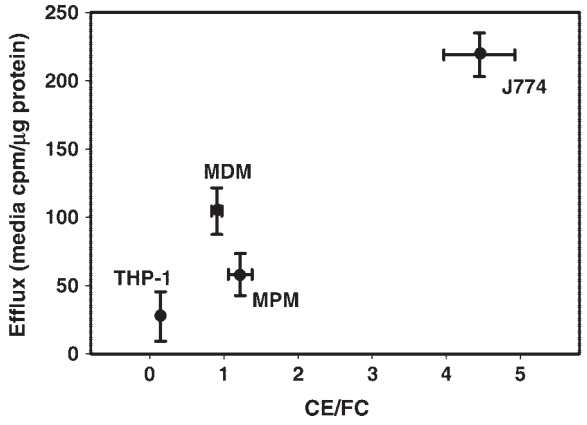


**Fig. 1.** Cholesterol efflux correlates with the cholesteryl ester/free cholesterol (CE/FC) ratio. A standard cholesterol efflux assay was performed on J774, MPM, THP-1, and MDM cells. Total cellular lipids were separated by thin-layer chromatography, and the CE/FC ratio was calculated. The correlation is plotted as cholesterol efflux (media cpm/μg cell protein ± SD) as a function of CE/FC (± SD).

Western blotting of ABCA1 demonstrated that the efflux levels approximately correlated with the ABCA1 expression level, and that treatment with cAMP, CTX, or 9cRA increased ABCA1 expression (blot shown is representative of multiple experiments). In MPMs, the relative effects of cAMP, CTX, and 9cRA were identical to those in J774 cells, except that the efflux was not as robust (Fig. 2B). On the other hand, ABCA1 expression levels were only increased by 9cRA treatment and only modestly with cAMP or CTX treatment. In THP-1 cells (Fig. 2C) and MDMs (Fig. 2D), there was no effect of cAMP or CTX on efflux. Interestingly, there was a significant increase in efflux upon 9cRA treatment, and an additive effect of cAMP and 9cRA. ABCA1 expression levels were increased with 9cRA treatment, whereas cAMP and CTX modestly increased ABCA1 expression. This result clearly shows a unique regulation of efflux by cAMP and 9cRA in human macrophages, different from that in murine macrophages, and suggests that cAMP regulates efflux by a mechanism other than by stimulating expression.

### Effect of cAMP on PKA activity

To investigate the effect of cAMP and whether its effect is mediated through cAMP-dependent PKA, we utilized H89, a specific PKA inhibitor, at increasing concentrations (data not shown). We have completed time courses and concentration curves in each cell type in separate experiments and determined the best working conditions for H89. In J774 cells, H89 was only mildly effective at inhibiting efflux when included with a 12 h pretreatment (**Fig. 3A**). When used in combination with cAMP, H89 was ineffective. Western blotting of ABCA1 demonstrated that the efflux levels approximately correlated with the ABCA1 expression level, and that treatment with cAMP increased ABCA1 expression but H89 had no effect (blot shown is representative of multiple experiments). However, in the other cell types, H89 had an intriguing effect. In MPMs, H89 had the ability to inhibit the cAMP-stimulated efflux (pretreated for 12 h or added only during efflux), but H89 had no inhibitory effect on basal efflux in the absence of cAMP (Fig. 3B). On the other hand, Western blotting of ABCA1 demonstrated that 12 h treatment with H89 actually increased ABCA1 expression, in addition to the enhancement of expression due to cAMP (blot shown is representative of multiple experiments). In THP-1 cells (Fig. 3C) and MDMs (Fig. 3D), H89 had the ability to significantly reduce the basal level of efflux when added during the efflux period in the absence or presence of cAMP. Western blotting of ABCA1 demonstrated that H89 treatment did not have an effect on ABCA1 expression (blot shown is representative of multiple experiments). It should be noted that in the experiments utilizing cAMP and H89 (Figs. 2, 3), phospholipid efflux experiments were performed in parallel and the relative efflux values were completely in concurrence (data not shown).

### Effect of Gi on cholesterol efflux

PTX, the inhibitor of the Gi, and mastoparan, an activator of Gi, were introduced to the media of J774 cells at 24 h,

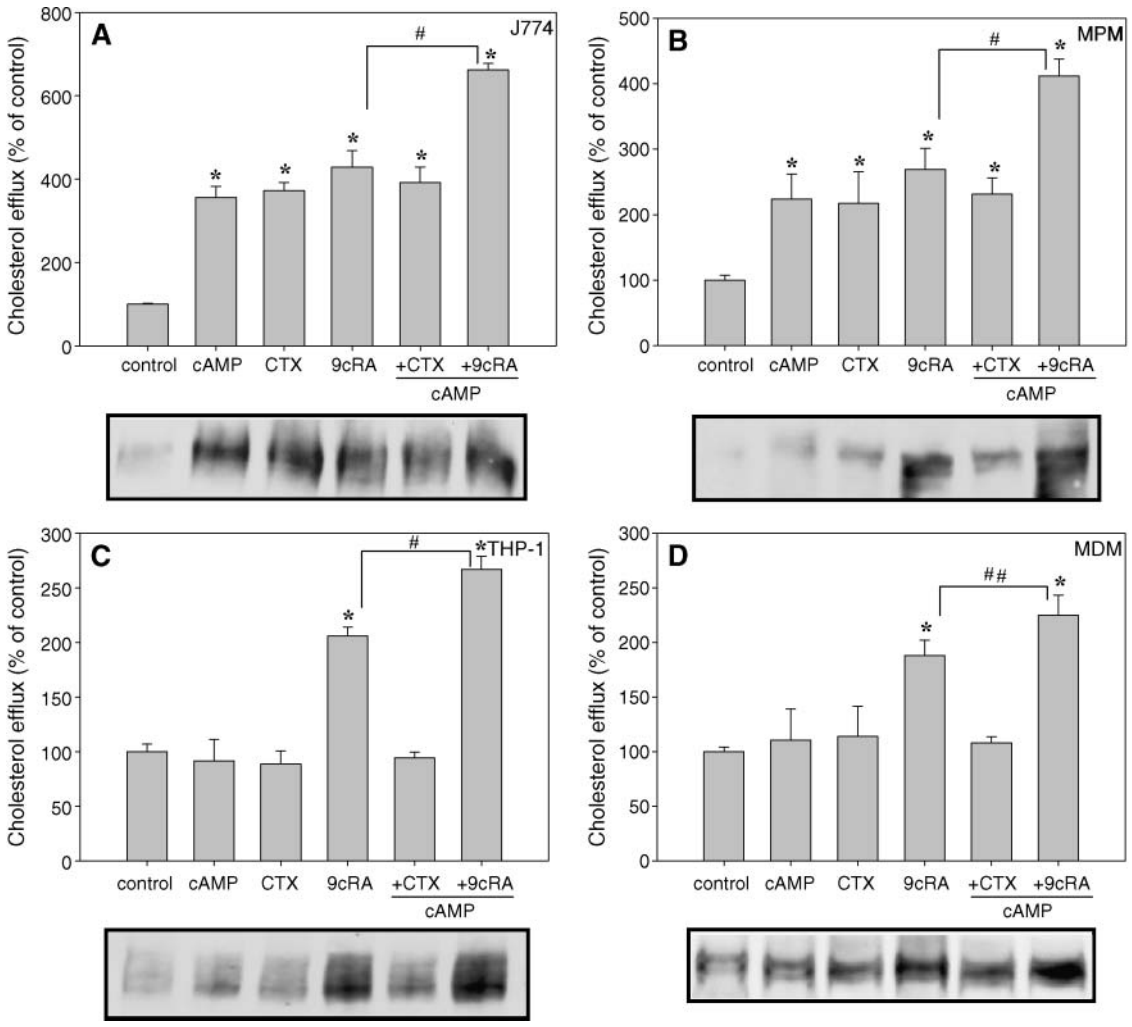


**Fig. 2.** Independent effect of cyclic AMP (cAMP) on ABCA1-mediated efflux. J774 (A), MPM (B), THP-1 (C), and MDM (D) cells were treated with cAMP (150 μM), CTX (2 μg/ml), and 9cRA (10 μM) 12 h prior to and during efflux. Cells were then incubated with or without apolipoprotein A-I (apoA-I) (50 μg/ml) in Dulbecco's modified Eagle's medium (DMEM)/BSA for 5 h. ApoA-I-specific [$^3$H]cholesterol efflux was calculated, and apoA-I-specific efflux in the absence of any treatment was normalized to 100% (see Fig. 1 for average values, cpm media/μg cell protein). Each individual experiment was performed in quadruplicate, and the average (±SD) of four independent experiments is presented here. * $P < 0.01$ for this condition compared with control; # $P < 0.01$ between the two indicated conditions; ## $P < 0.05$ between the two indicated conditions. Western blot of ABCA1 for each treatment condition has been normalized to total cell protein and correlates with the efflux bars directly above. Quantification of bands for three independent experiments was compared with control (1.0-fold) and represents the fold change. J774 cells: cAMP, 6.4 ± 0.4; CTX, 6.5 ± 0.2; 9cRA, 6.2 ± 0.2; cAMP + CTX, 6.1 ± 0.2; cAMP + 9cRA, 7.6 ± 0.6. MPMs: cAMP, 2.7 ± 0.9; CTX, 3.2 ± 0.7; 9cRA, 6.1 ± 0.7; cAMP + CTX, 3.3 ± 0.4; cAMP + 9cRA, 7.6 ± 0.6. THP-1 cells: cAMP, 1.4 ± 0.4; CTX, 1.4 ± 0.3; 9cRA, 4.4 ± 0.4; cAMP + CTX, 1.5 ± 0.3; cAMP + 9cRA, 6.3 ± 0.8. MDMs: cAMP, 1.1 ± 0.1; CTX, 1.2 ± 0.1; 9cRA, 2.3 ± 0.6; cAMP + CTX, 1.3 ± 0.2; cAMP + 9cRA, 3.1 ± 0.7.

12 h, 2 h, and 0 h prior to efflux at varying concentrations to determine the optimal conditions (data not shown). For PTX, 12 h pretreatment gave the largest increase in efflux, whereas treatment with mastoparan only during the efflux period was most effective. Consistent with the hypothesis that Gi is involved in efflux regulation, mastoparan and PTX had opposing effects. Mastoparan inhibited close to 40% and PTX stimulated close to 60% (**Fig. 4**). The effects of these two drugs cancelled each other out following a combined treatment and returned the net cholesterol efflux to the initial, control level. Mastoparan showed definite signs of inhibition, inasmuch as it decreased the efflux by 25% even in the presence of cAMP (data not shown). Western blotting of ABCA1 demonstrated that both mastoparan and PTX treatment increased ABCA1 expression (blot shown is representative of multiple experiments). In the other cells, there was no significant effect of PTX or mastoparan at any time point (data not shown).

### Effect of anisomycin on cholesterol efflux

Anisomycin strongly activates Jun N-terminal kinase, also known as stress-activated protein kinase (JNK/SAPK) activity and, to a lesser extent, p38 [reactivating kinase (RK)] activity and mitogen-activated protein kinases (MAPKs). Anisomycin was introduced to J774 cells at 200 nM, inhibiting cholesterol efflux by approximately 50% (**Fig. 5**). A similar effect was also observed in THP-1 cells. Interest-

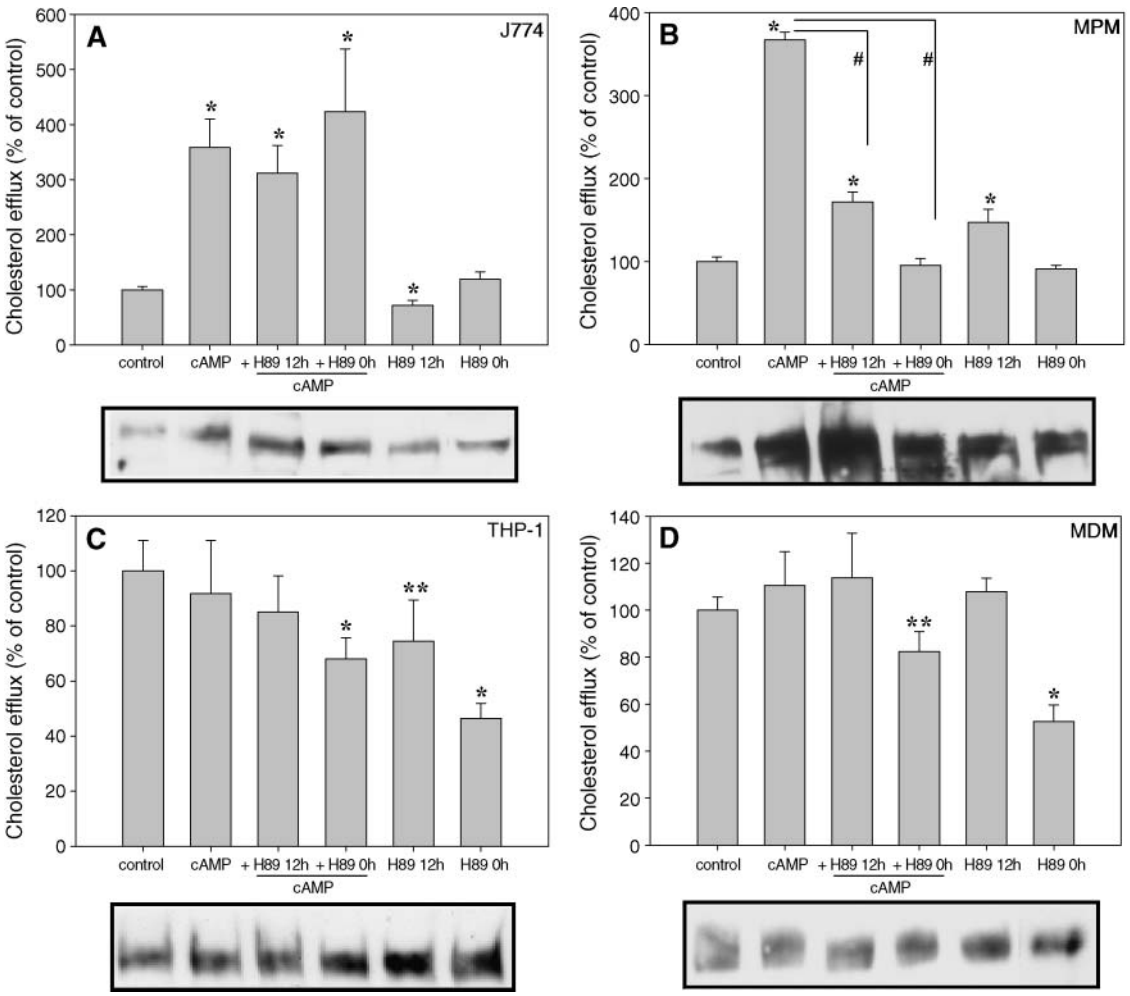


**Fig. 3.** PKA inhibitor (H89) inhibits only cAMP-stimulated efflux in MPMs and basal efflux in THP-1 and MDM cells. J774 (A), MPM (B), THP-1 (C), and MDM (D) cells were treated with cAMP (150 μM) or H89 (5 μM) for either 12 h prior to and during efflux or just during efflux. Cells were then incubated with or without apoA-I (50 μg/ml) in DMEM/BSA for 5 h. ApoA-I-specific [$^3$H]cholesterol efflux was calculated, and apoA-I-specific efflux in the absence of any treatment was normalized to 100%. Each individual experiment was performed in quadruplicate, and the average (±SD) of four experiments is presented here. * $P < 0.01$ for this condition compared with control; ** $P < 0.05$ for this condition compared with control; # $P < 0.01$ between the two indicated conditions. Western blot of ABCA1 for each treatment condition has been normalized to total cell protein and correlates with the efflux bars directly above. Quantification of bands for three independent experiments was compared with control (1.0-fold) and represents the fold change. J774 cells: cAMP, 5.9 ± 0.5; cAMP + H89 12 h, 5.7 ± 0.5; cAMP + H89 0 h, 6.1 ± 0.3; H89 12 h, 1.3 ± 0.1; H89 0 h, 1.0 ± 0.3. MPMs: cAMP, 3.3 ± 0.3; cAMP + H89 12 h, 6.1 ± 0.7; cAMP + H89 0 h, 3.0 ± 0.2; H89 12 h, 1.5 ± 0.1; H89 0 h, 1.2 ± 0.3. THP-1 cells: cAMP, 1.0 ± 0.1; cAMP + H89 12 h, 1.1 ± 0.1; cAMP + H89 0 h, 1.2 ± 0.2; H89 12 h, 1.5 ± 0.2, H89 0 h, 1.4 ± 0.2. MDMs: cAMP, 1.0 ± 0.1; cAMP + H89 12 h, 1.0 ± 0.1; cAMP + H89 0 h, 1.1 ± 0.2; H89 12 h, 1.3 ± 0.2; H89 0 h, 1.3 ± 0.2.

ingly, anisomycin stimulated efflux in MPMs (400%) and MDMs (50%), both primary cells. At this low concentration, we observed no induction of apoptosis (data not shown), but a significant effect on cholesterol efflux. This result illustrates an important difference between cell lines and primary cells of mouse and human origin. Western blotting of ABCA1 demonstrated that treatment with anisomycin had no effect on ABCA1 expression (data not shown).

### Role of PKC in lipid efflux

PKC activators DOG and PMA, and the PKC inhibitor, CalC, were used to assess the involvement of PKC in lipid efflux. We have completed time courses and concentration curves with each drug in each cell type in separate experiments and determined the best working conditions for each agent. DOG, PMA, and CalC had no significant effect in J774 cells and MDMs. The same lack of an effect was seen even in the presence of cAMP (data not shown). However, in MPM cells (**Fig. 6A**), the PKC activators caused stimulation of cholesterol efflux, and the PKC inhibitors caused an inhibition of cholesterol efflux. The respective inhibition and stimulation of these drugs was even more pronounced in the presence of cAMP, where CalC inhibited the cAMP effect by about 50% and DOG induced an additive effect with cAMP. This result suggests that PKA and PKC activation of efflux is at least partially independent. When added together, CalC and DOG mutually canceled each others' effects. Western blotting of ABCA1

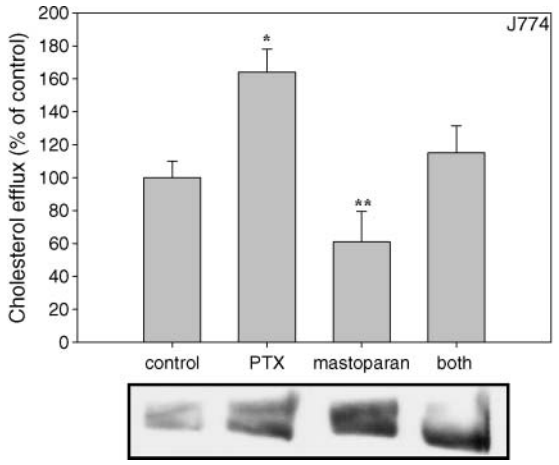


**Fig. 4.** Mastoparan and pertussis toxin (PTX) have opposing effects on cholesterol efflux in J774 cells. J774 cells were treated with PTX (100 ng/ml) for 12 h prior to and during efflux, and cells were treated with mastoparan (10 μM) only during the efflux period. Cells were then incubated with or without apoA-I (50 μg/ml) in DMEM/BSA for 5 h. ApoA-I-specific [$^3$H]cholesterol efflux was calculated, and apoA-I-specific efflux in the absence of any treatment was normalized to 100%. Each individual experiment was performed in quadruplicate, and the average (±SD) of three experiments is presented here. * $P < 0.01$ for this condition compared with control; ** $P < 0.05$ for this condition compared with control. Western blot of ABCA1 for each treatment condition has been normalized to total cell protein and correlates with the efflux bars directly above. Quantification of bands for three independent experiments was compared with control (1.0-fold) and represents the fold change. J774 cells: PTX, 2.8 ± 0.8; mastoparan, 3.2 ± 1.0; both, 2.6 ± 0.5.

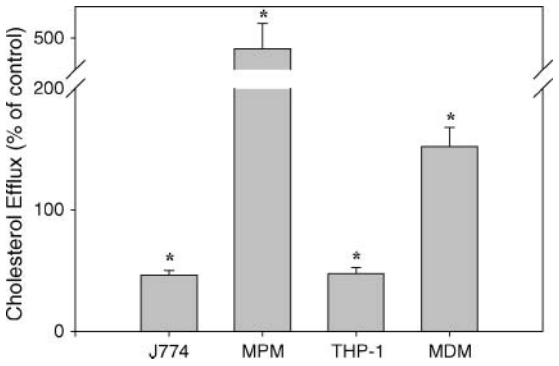


**Fig. 5.** Anisomycin stimulates efflux in primary macrophages and inhibits efflux in transformed macrophage cell lines. J774, MPM, THP-1, and MDM cells were treated with anisomycin (200 nM) 1/2 h prior to efflux. Then cells were incubated with or without apoA-I (50 μg/ml) in DMEM/BSA for 5 h. ApoA-I-specific [$^3$H]cholesterol efflux was calculated, and apoA-I-specific efflux in the absence of any treatment for each cell type was normalized to 100%. Each individual experiment was performed in quadruplicate, and the average (±SD) of three experiments is presented here. * $P < 0.01$ for this condition compared with control.

demonstrated that treatment with CalC, PMA, and DOG had no effect on ABCA1 expression (blot shown is representative of multiple experiments). In THP-1 cells, under the same treatment conditions, these effects were also observed (Fig. 6B), although to a lesser extent. In THP-1 cells, Western blotting of ABCA1 demonstrated that treatment with CalC, PMA, and DOG had no effect on ABCA1 expression (blot shown is representative of multiple experiments).

To address the hypothesis that PKA primarily regulates ABCA1 and phospholipid and cholesterol efflux, whereas PKC affects only cholesterol efflux, we examined phospholipid and cholesterol efflux in MPM (**Fig. 7A**) and THP-1 (Fig. 7B) cells in the presence of the PKC agents. Phospholipid efflux was unaffected by CalC and DOG, whereas cholesterol efflux was sensitive to all treatments. Glyburide treatment acted as the positive control for inhibition of both phospholipid and cholesterol efflux. As a result, we can demonstrate that phospholipid and cholesterol efflux can be functionally separated in MPM and THP-1 cells, and that PKC effectors affect only cholesterol efflux in these two cell types. The comparison of all the results for each cell type is shown in **Table 1**.

## DISCUSSION

ABCA1 expression can be regulated by cellular cholesterol levels and by ligand stimulation of the LXR/RXR response element in the ABCA1 gene. cAMP is also potentially capable of stimulating ABCA1 transcription, but by a mechanism distinct from LXR/RXR. No cAMP response element in the proximal region of the ABCA1 gene has thus far been found. Cavelier and colleagues showed that human ABCA1 gene expression responded differently to cAMP than mouse ABCA1 did (5). Some studies showed that J774 cells and MPMs display cAMP-stimulated ABCA1 gene transcription and surface expression in the absence of cholesterol loading (32, 47). Our results showed that cAMP significantly stimulated cholesterol efflux in mouse J774 cells and MPMs, but not in human THP-1 cells or MDMs, confirming some previous observations (12, 47, 48). Contreras and Lasuncion (12) argued that the lack of a cAMP effect in human macrophages was due to the apparent absence of hormone-sensitive lipase in human macrophages, but this point is controversial (49–51). We extended these results by examining the effects of CTX and 9cRA. In mouse cells, CTX stimulated efflux to the same level as did cAMP, and in human cells, CTX was equally ineffective as cAMP (Fig. 2). When cAMP and CTX were added together, no additional efflux was observed, indicating that they were working through the same pathway. On the other hand, 9cRA increased efflux and ABCA1 expression in all cells, including the human macrophages, even though there was no effect of cAMP. The combination of 9cRA and cAMP was additive in all cells, even in the human macrophages that had no response to cAMP when added alone. In J774 cells, cAMP and CTX did induce expression of ABCA1, and H89 had almost no effect on efflux, indicating that the effect of cAMP in J774 cells is due to induced expression of ABCA1. In MPMs, ABCA1 expression was moderately induced by cAMP but H89 was able to inhibit the cAMP-inducible efflux. Therefore, the cAMP-inducible phosphorylation of ABCA1 by PKA is a component of the efflux

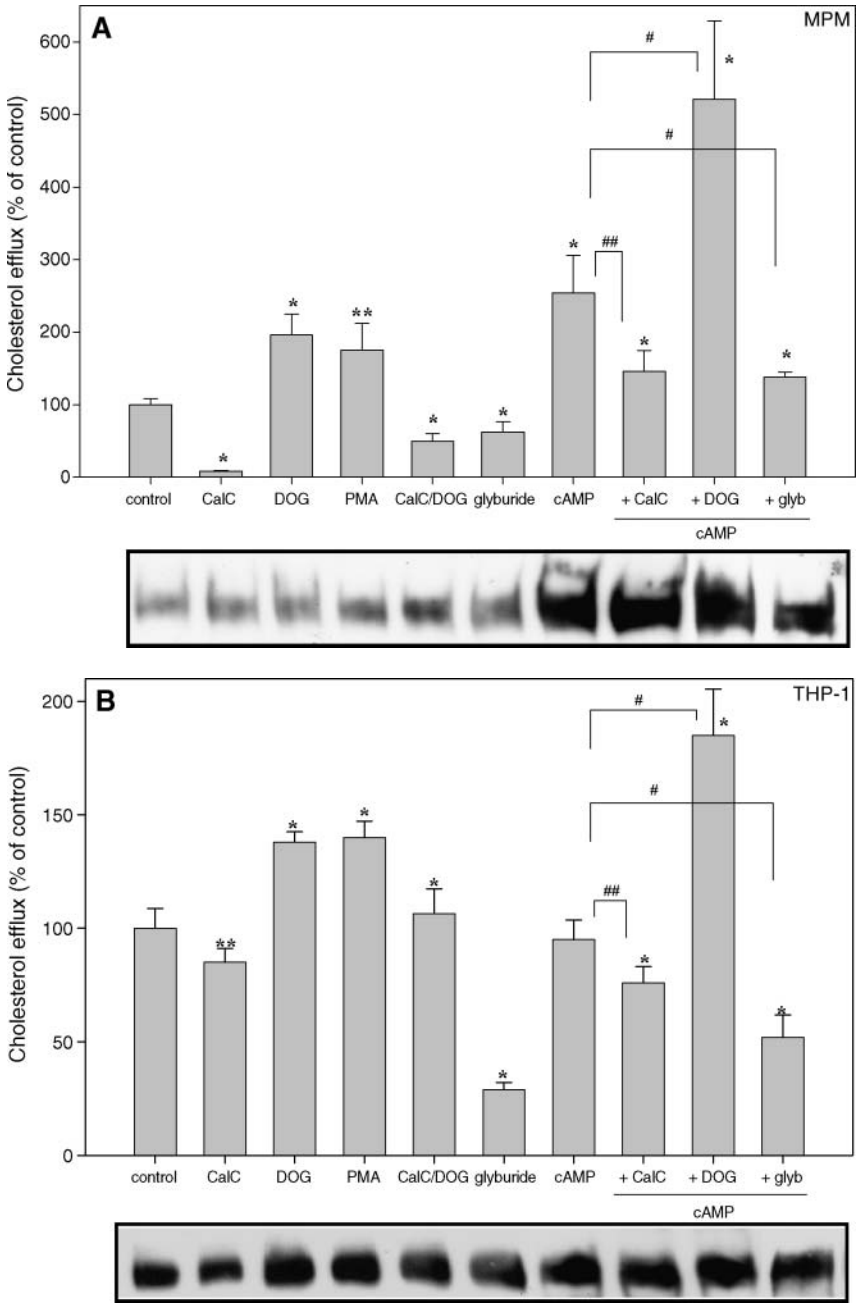


**Fig. 6.** PKC effectors regulate cholesterol efflux. MPM (A) and THP-1 (B) cells were treated with cAMP (150 μM) 12 h prior to and during efflux. Cells were treated with dioctanoylglycerol (DOG) (200 μM), phorbol 12-myristate 13-acetate (PMA) (100 nM), and calphostin C (CalC) (50 nM) 3 h prior to efflux. Cells were then incubated with or without apoA-I (50 μg/ml) in DMEM/BSA for 5 h. ApoA-I-specific [$^3$H]cholesterol efflux was calculated, and apoA-I-specific efflux in the absence of any treatment was normalized to 100%. Each individual experiment was performed in quadruplicate, and a representative of four experiments is presented here. * $P < 0.01$ for this condition compared with control; ** $P < 0.05$ for this condition compared with control; # $P < 0.01$ between the two indicated conditions; ## $P < 0.05$ between the two indicated conditions. Western blot of ABCA1 for each treatment condition has been normalized to total cell protein and correlates with the efflux bars directly above. Quantification of bands for three independent experiments was compared with control (1.0-fold) and represents the fold change. MPM cells: CalC, 1.2 ± 0.2; DOG, 1.0 ± 0.2; PMA, 1.1 ± 0.2; CalC/DOG, 1.5 ± 0.3; glyburide, 1.3 ± 0.3; cAMP, 3.7 ± 0.5; cAMP + CalC, 4.1 ± 0.6; cAMP + DOG, 3.6 ± 0.2; cAMP + glyburide, 3.4 ± 0.3. THP-1 cells: CalC, 1.0 ± 0.3; DOG, 1.2 ± 0.4; PMA, 1.1 ± 0.3; CalC/DOG, 1.1 ± 0.3; glyburide, 1.0 ± 0.2; cAMP, 1.3 ± 0.4; cAMP + CalC, 1.3 ± 0.3; cAMP + DOG, 1.4 ± 0.4; cAMP + glyburide, 1.2 ± 0.3.

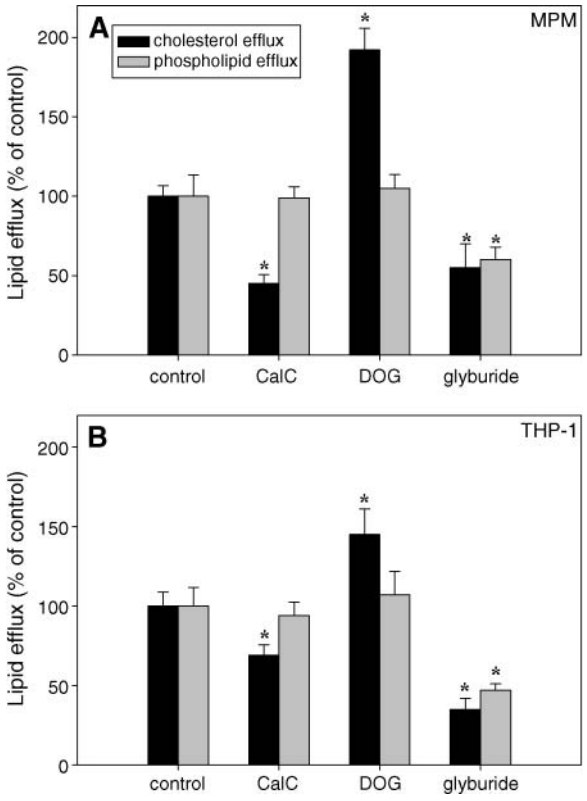


**Fig. 7.** PKC effectors regulate cholesterol efflux but do not affect phospholipid efflux. MPM (A) and THP-1 (B) cells were treated with DOG (200 μM), CalC (50 nM) 3 h prior to efflux or glyburide (100 μM) 1/2 h prior to efflux. Cells were then incubated with or without apoA-I (50 μg/ml) in DMEM/BSA for 5 h. ApoA-I and associated lipids were immunoprecipitated from the media. ApoA-I-specific [$^3$H]cholesterol efflux and [$^3$H]phospholipid efflux (cpm/mg cell protein) were calculated, and apoA-I-specific efflux in the absence of any treatment was normalized to 100%. Each individual experiment was performed in triplicate, and a representative experiment is presented here. * $P < 0.01$ for this condition compared with control.

machinery in MPMs. In THP-1 and MDM cells, where cAMP alone had no effect on efflux, H89 was effective at reducing the basal level of efflux in the absence of cAMP and less significantly in the presence of cAMP. The H89 effect in THP-1 and MDM cells was more evident when H89 was added during the efflux period (no pretreatment) suggesting that the role of cAMP was more in functional regulation by PKA-dependent phosphorylation than in transcription. We propose that in THP-1 and MDM cells, where cAMP alone has no effect, the basal cholesterol-loaded state already has an optimally upregulated PKA and ABCA1, which can be inhibited by H89. Only upon induction of expression of ABCA1 by another means (in this case 9cRA treatment) can we further promote efflux by cAMP, probably by both enhancement of transcription [Western blot (Fig. 2)] and PKA-dependent phosphorylation of ABCA1 (sensitive to H89). Therefore, cAMP regulates cholesterol efflux predominantly by activation of PKA in MPMs, THP-1 cells, and MDMs, but in J774 cells, cAMP regulates cholesterol efflux by induction of expression of ABCA1.

Previously, it was found that PTX could induce a TD-like pattern of formation of lipids in fibroblasts, suggesting the involvement of Gi in the regulation of lipid efflux (30). PTX was used to inhibit Gi, thereby preventing Gi from inhibiting adenylate cyclase. As such, the effect of PTX is very subtle in comparison to CTX treatment. However, PTX also inhibited cholesterol efflux in the presence of cAMP, bypassing the regulation of adenylate cyclase, suggesting that PTX may act at another site, perhaps in the regulation of PLC and PLD (30).

Anisomycin strongly activates JNK/SAPK activity and, to a lesser extent, p38 (RK) activity and MAPKs (52, 53). Anisomycin treatment stimulated cholesterol efflux in human skin fibroblasts (54). Anisomycin introduced to J774 and THP-1 cells inhibited cholesterol efflux approximately 40–50% (Fig. 5). On the other hand, cholesterol efflux was stimulated in MPM (350%) and MDM (50%) cells. In these cases, the transformed cell lines were inhibited and primary cells were stimulated by anisomycin. Previous work showed that cAMP significantly stimulates efflux in immortalized fibroblasts, but not as strongly as in primary fibroblasts (2, 55), demonstrating that transformation of cells alters the regulation and metabolic state of cells. These observations lead us to propose that the JNK/SAPK pathways are alternatively regulated in transformed cells and primary cells, and the JNK/SAPK pathway may play a role in cholesterol efflux. Recently, Witting, Maiorano, and Davidson demonstrated that ceramide increased ABCA1-dependent cholesterol efflux without inducing apoptosis (56), with supporting evidence that ceramide may be acting through JNK/SAPK (57, 58).

Apolipoprotein-mediated lipid efflux involves the interaction of apoA-I with the cell surface, and then ABCA1 translocates phospholipid and possibly cholesterol at the plasma membrane to apoA-I. The role of ABCA1 in cholesterol efflux and the possible separation of functional phospholipid and cholesterol efflux is a matter under current investigation and may be cell specific (34, 35, 59). ApoA-I/phospholipid complexes may trigger downstream signaling, causing the release of intracellular stores of cholesterol, and subsequently promote cholesterol efflux in a second step (34, 35, 60). The nature of these intracellular cholesterol stores is currently unclear, although some recent work suggests that cholesterol from the late endosome/lysosome (61) or endosomal recycling compartment (62), as well as newly synthesized cholesterol and stored cholesteryl ester (63), may be major sources. The observation that cholesterol efflux levels correlate with the CE/FC ratio (Fig. 1) also suggests that in these macrophages, the ACAT-accessible pool serves as a major source of cholesterol for efflux.

Mendez, Oram, and Bierman suggested that the activation of PKC results in mobilization and translocation of intracellular cholesterol from its storage location to the cell surface (64). This result was later confirmed by Li, Tsujita, and Yokoyama (32) and Drobnik et al. (28). Activation of PKC by phorbol esters resulted in activation of efflux to apoA-I or HDL (27, 32, 64–66). It is believed that the PKC cascade involves the PC-specific PLC-mediated breakdown

TABLE 1. Summary of relative effects of different agents on phospholipid and cholesterol efflux in four different macrophagic cell types[a]

| | J774 | | MPM | | THP-1 | | MDM | |
|---|---|---|---|---|---|---|---|---|
| Agents | PL Efflux | FC Efflux | PL Efflux | FC Efflux | PL Efflux | FC Efflux | PL Efflux | FC Efflux |
| cAMP | 225 ± 23 | 356 ± 27 | 284 ± 47 | 224 ± 37 | 102 ± 22 | 92 ± 19 | 110 ± 20 | 111 ± 29 |
| 9-cRA | 323 ± 45 | 428 ± 40 | 302 ± 39 | 269 ± 32 | 241 ± 25 | 206 ± 8 | 239 ± 41 | 188 ± 14 |
| CTX | 244 ± 62 | 372 ± 20 | 267 ± 42 | 217 ± 48 | 103 ± 9 | 89 ± 12 | 112 ± 19 | 114 ± 28 |
| H89 0 h | 112 ± 21 | 119 ± 13 | 118 ± 22 | 91 ± 4 | 51 ± 13 | 46 ± 15 | 63 ± 10 | 53 ± 7 |
| H89 12 h | 109 ± 8 | 72 ± 9 | 124 ± 29 | 147 ± 16 | 82 ± 9 | 74 ± 6 | 98 ± 8 | 108 ± 6 |
| CalC | 90 ± 15 | 91 ± 9 | 99 ± 7 | 8 ± 1 | 97 ± 6 | 85 ± 6 | 95 ± 11 | 97 ± 15 |
| DOG | 107 ± 17 | 106 ± 14 | 105 ± 9 | 196 ± 29 | 103 ± 5 | 138 ± 5 | 102 ± 12 | 109 ± 12 |
| PMA | 104 ± 16 | 112 ± 11 | 110 ± 10 | 175 ± 38 | 106 ± 17 | 140 ± 7 | 114 ± 23 | 113 ± 25 |
| Glyburide | 55 ± 8 | 53 ± 11 | 48 ± 7 | 63 ± 14 | 36 ± 7 | 29 ± 3 | 44 ± 5 | 56 ± 16 |
| PTX | nd | 164 ± 14 | nd | 98 ± 12 | nd | 113 ± 20 | nd | 95 ± 7 |
| Mastoparan | nd | 61 ± 19 | nd | 91 ± 7 | nd | 92 ± 17 | nd | 89 ± 14 |
| Anisomycin | nd | 46 ± 4 | nd | 483 ± 40 | nd | 47 ± 6 | nd | 152 ± 16 |

[a] J774, MPM, THP-1, and MDM cells were prepared as described in Experimental Procedures. Mouse J774 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Mouse peritoneal macrophages (MPMs) were obtained by peritoneal lavage from Swiss-Webster mice and maintained in DMEM/10% FBS media for 72 h prior to cholesterol loading. Human THP-1 cells were grown in suspension in RPMI media supplemented with 10% FBS, 1% P/S, 1% L-glutamine, and 5 mM β-mercaptoethanol. They were differentiated by treatment with phorbol myristyl acetate (PMA) (10 nM) for 72 h prior to the experiment. Human monocyte-derived macrophages (MDMs) were prepared following the procedure of Cohen et al. (44). The cells were maintained in culture for 12 days to allow the monocytes to differentiate into macrophages. Cholesterol loading and efflux assays were performed according to the Experimental Procedures.

CalC, calphostin C; cAMP, cyclic AMP; 9-cRA, 9-*cis*-retinoic acid; CTX, cholera toxin; DOG, 1,2-dioctanoylglycerol; FC, free cholesterol; nd, not determined; PL, phospholipid; PTX, pertussis toxin.

of PC into DAG, which is responsible for the activation of PKC and phosphorylation of ABCA1 (29). In SMCs, Li, Tsujita, and Yokoyama used PMA to activate PKC and thereby induced a significant increase in cholesterol efflux (31, 32). Recently, Haidar et al. performed a study in human fibroblasts to determine the effects of PKC activation and inhibition on cholesterol efflux (26). The evidence that PKC activators can stimulate cholesterol efflux in Tangier fibroblasts suggests that cholesterol efflux is partially independent of ABCA1 and that ABCA1 may not be the only target of PKC. This finding also suggests the very interesting possibility that phospholipid and cholesterol efflux can be independently regulated. In this case, Li, Tsujita, and Yokoyama demonstrated that PKC inhibition had an effect only on cholesterol efflux, and not on phospholipid efflux, in MPMs (32). Our results are strongly in support of this observation. Recently, Yamauchi et al. demonstrated that PKC inhibitors could inhibit phospholipid efflux to a small but significant extent in WI-38 fibroblasts (29). More recently, the same authors confirmed a difference between WI-38 and L929 fibroblasts, and suggested that PKC may independently regulate cholesterol efflux in L929 cells (33). Other evidence suggests that cholesterol efflux may be coincident with phospholipid efflux and is mediated by ABCA1 (59). Our results suggest that both PKA and PKC can stimulate cholesterol efflux and that the mechanisms are at least partially independent. PKC agents did not affect phospholipid efflux in MPM and THP-1 cells, and the combination of a PKC-stimulatory agent (DOG) and cAMP resulted in an additive stimulatory effect on efflux. Therefore, our results suggest that in the macrophages described in this study, PKA regulates both phospholipid and cholesterol efflux, whereas PKC regulates a separate step of cholesterol efflux. This action of PKC in mobilizing stored cholesteryl ester would be consistent with our results, although there have been no reports of activation by PKC on hormone-sensitive lipase or cholesteryl ester hydrolase. Our data do not rule out the possibility that PKC can also regulate ABCA1 directly, by phosphorylation.

In this paper, we have made many novel comments about macrophage lipid efflux. We have defined the effects of cAMP in four commonly used macrophage cells. In MPMs, cAMP regulates ABCA1 by modulation of expression and phosphorylation by PKA, whereas in J774 cells, cAMP only affects expression of ABCA1. PKC predominantly induces cholesterol efflux, but not phospholipid efflux, independent of an induction of ABCA1 expression in MPM and THP-1 cells. On the basis of these studies, uninduced MPMs represent the best model for mouse macrophages. In MPMs, ABCA1 expression was responsive to cAMP, but all cAMP-inducible efflux was inhibitable by H89, a PKA inhibitor. As well, MPM cholesterol efflux was modulated by PKC agents. For human macrophages, THP-1 cells and MDMs were equally reliable in terms of responsiveness to treatments, but the variability inherent in multiple donors for MDMs and the extended period in culture makes MDMs more difficult. As part of the present literature review, it has become evident that cell type (including primary and transformed cells) can play a very important role in determining which pathways regulate lipid efflux. In fact, the conflicting data about regulation of lipid efflux present in the literature may predominantly be explained by the choice of cell model. This work has highlighted differences even between macrophages. In any case, relevant cell models must be considered to attain appropriate information about ABCA1 regulation and lipid efflux.

The authors would like to thank Dr. Bassam Haidar for critical reading of the manuscript. The authors would also like to thank Vivian Franklin for expert technical assistance.

## REFERENCES


1. Langmann, T., M. Porsch-Ozcurumez, S. Heimerl, M. Probst, C. Moehle, M. Taher, H. Borsukova, D. Kielar, W. E. Kaminski, E. Dittrich-Wengenroth, et al. 2002. Identification of sterol-independent regulatory elements in the human ATP-binding cassette transporter A1 promoter: role of Sp1/3, E-box binding factors, and an oncostatin M-responsive element. *J. Biol. Chem.* **277:** 14443–14450.
2. Lawn, R. M., D. P. Wade, M. R. Garvin, X. B. Wang, K. Schwartz, J. G. Porter, J. J. Seilhamer, A. M. Vaughan, and J. F. Oram. 1999. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *J. Clin. Invest.* **104:** R25–R31.
3. Schwartz, K., R. M. Lawn, and D. P. Wade. 2000. ABC1 gene expression and ApoA-I-mediated cholesterol efflux are regulated by LXR. *Biochem. Biophys. Res. Commun.* **274:** 794–802.
4. Costet, P., Y. Luo, N. Wang, and A. R. Tall. 2000. Sterol-dependent transactivation of the human ABC1 promoter by LXR/RXR. *J. Biol. Chem.* **275:** 28240–28245.
5. Cavelier, L. B., Y. Qiu, J. K. Bielicki, V. Afzal, J. F. Cheng, and E. M. Rubin. 2001. Regulation and activity of the human ABCA1 gene in transgenic mice. *J. Biol. Chem.* **276:** 18046–18051.
6. Bernard, D. W., A. Rodriguez, G. H. Rothblat, and J. M. Glick. 1991. cAMP stimulates cholesteryl ester clearance to high density lipoproteins in J774 macrophages. *J. Biol. Chem.* **266:** 710–716.
7. Bortnick, A. E., G. H. Rothblat, G. Stoudt, K. L. Hoppe, L. J. Royer, J. McNeish, and O. L. Francone. 2000. The correlation of ABC1 mRNA levels with cholesterol efflux from various cell lines. *J. Biol. Chem.* **275:** 28634–28640.
8. Oram, J. F., R. M. Lawn, M. R. Garvin, and D. P. Wade. 2000. ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J. Biol. Chem.* **275:** 34508–34511.
9. Zheng, P., A. Horwitz, C. A. Waelde, and J. D. Smith. 2001. Stably transfected ABCA1 antisense cell line has decreased ABCA1 mRNA and cAMP-induced cholesterol efflux to apolipoprotein AT and HDL. *Biochim. Biophys. Acta.* **1534:** 121–128.
10. Stein, O., M. Ben Naim, Y. Dabach, G. Hollander, and Y. Stein. 2002. Macrophage cholesterol efflux to free apoprotein A-I in C3H and C57BL/6 mice. *Biochem. Biophys. Res. Commun.* **290:** 1376–1381.
11. Kritharides, L., A. Christian, G. Stoudt, D. Morel, and G. H. Rothblat. 1998. Cholesterol metabolism and efflux in human THP-1 macrophages. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1589–1599.
12. Contreras, J. A., and M. A. Lasuncion. 1994. Essential differences in cholesteryl ester metabolism between human monocyte-derived and J774 macrophages. Evidence against the presence of hormone-sensitive lipase in human macrophages. *Arterioscler. Thromb.* **14:** 443–452.
13. Abe-Dohmae, S., S. Suzuki, Y. Wada, H. Aburatani, D. E. Vance, and S. Yokoyama. 2000. Characterization of apolipoprotein-mediated HDL generation induced by cAMP in a murine macrophage cell line. *Biochemistry.* **39:** 11092–11099.
14. Martinez, L., B. Agerholm-Larsen, N. Wang, W. Chen, and A. R. Tall. 2003. Phosporylation of a pest sequence in ABCA1 promotes calpain degradation and is reversed by ApoA-I. *J. Biol. Chem.* **278:** 37368–37374.
15. Wang, N., and A. R. Tall. 2003. Regulation and mechanisms of ATP-binding cassette transporter A1-mediated cellular cholesterol efflux. *Arterioscler. Thromb. Vasc. Biol.* **23:** 1178–1184.
16. Feng, B., and I. Tabas. 2002. ABCA1-mediated cholesterol efflux is defective in free cholesterol-loaded macrophages. Mechanism involves enhanced ABCA1 degradation in a process requiring full NPC1 activity. *J. Biol. Chem.* **277:** 43271–43280.
17. Becq, F., Y. Hamon, A. Bajetto, M. Gola, B. Verrier, and G. Chimini. 1997. ABC1, an ATP binding cassette transporter required for phagocytosis of apoptotic cells, generates a regulated anion flux after expression in Xenopus laevis oocytes. *J. Biol. Chem.* **272:** 2695–2699.
18. Haidar, B., M. Denis, L. Krimbou, M. Marcil, and J. Genest, Jr. 2002. cAMP induces ABCA1 phosphorylation activity and promotes cholesterol efflux from fibroblasts. *J. Lipid Res.* **43:** 2087–2094.
19. See, R. H., R. A. Caday-Malcolm, R. R. Singaraja, S. Zhou, A. Silverston, M. T. Huber, J. Moran, E. R. James, R. Janoo, J. M. Savill, et al. 2002. Protein kinase A site-specific phosphorylation regulates ATP-binding cassette A1 (ABCA1)-mediated phospholipid efflux. *J. Biol. Chem.* **277:** 41835–41842.
20. Hokland, B. M., J. P. Slotte, E. L. Bierman, and J. F. Oram. 1993. Cyclic AMP stimulates efflux of intracellular sterol from cholesterol-loaded cells. *J. Biol. Chem.* **268:** 25343–25349.
21. Middleton, A., and B. Middleton. 1998. Elevation of cyclic AMP by iloprost and prostaglandin E1 increases cholesterol efflux and the binding capacity for high-density lipoproteins in human fibroblasts. *Biochim. Biophys. Acta.* **1391:** 117–132.
22. Osheroff, J. E., P. E. Visconti, J. P. Valenzuela, A. J. Travis, J. Alvarez, and G. S. Kopf. 1999. Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. *Mol. Hum. Reprod.* **5:** 1017–1026.
23. Lin, G., and K. E. Bornfeldt. 2002. Cyclic AMP-specific phosphodiesterase 4 inhibitors promote ABCA1 expression and cholesterol efflux. *Biochem. Biophys. Res. Commun.* **290:** 663–669.
24. Tang, C., A. M. Vaughan, and J. F. Oram. 2004. Janus kinase 2 modulates the apolipoprotein interactions with ABCA1 required for removing cellular cholesterol. *J. Biol. Chem.* **279:** 7622–7628.
25. Martinez, L. O., B. Agerholm-Larsen, N. Wang, W. Chen, and A. R. Tall. 2003. Phosphorylation of a pest sequence in ABCA1 promotes calpain degradation and is reversed by ApoA-I. *J. Biol. Chem.* **278:** 37368–37374.
26. Haidar, B., S. Mott, B. Boucher, C. Y. Lee, M. Marcil, and J. Genest, Jr. 2001. Cellular cholesterol efflux is modulated by phospholipid-derived signaling molecules in familial HDL deficiency/Tangier disease fibroblasts. *J. Lipid Res.* **42:** 249–257.
27. Rogler, G., B. Trumbach, B. Klima, K. J. Lackner, and G. Schmitz. 1995. HDL-mediated efflux of intracellular cholesterol is impaired in fibroblasts from Tangier disease patients. *Arterioscler. Thromb. Vasc. Biol.* **15:** 683–690.
28. Drobnik, W., C. Möllers, T. Resink, and G. Schmitz. 1995. Activation of phosphatidylinositol-specific phospholipase C in response to $HDL_3$ and LDL is markedly reduced in cultured fibroblasts from Tangier patients. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1369–1377.
29. Yamauchi, Y., M. Hayashi, S. Abe-Dohmae, and S. Yokoyama. 2003. Apolipoprotein A-I activates protein kinase C alpha signaling to phosphorylate and stabilize ATP binding cassette transporter A1 for the high density lipoprotein assembly. *J. Biol. Chem.* **278:** 47890–47897.
30. Walter, M., H. Reinecke, U. Gerdes, J. R. Nofer, G. Höbbel, U. Seedorf, and G. Assmann. 1996. Defective regulation of phosphatidylcholine-specific phospholipases C and D in a kindred with Tangier disease. Evidence for the involvement of phosphatidylcholine breakdown in HDL-mediated cholesterol efflux mechanisms. *J. Clin. Invest.* **98:** 2315–2323.
31. Li, Q. Q., and S. Yokoyama. 1995. Independent regulation of cholesterol incorporation into free apolipoprotein-mediated cellular lipid efflux in rat vascular smooth muscle cells. *J. Biol. Chem.* **270:** 26216–26223.
32. Li, Q. Q., M. Tsujita, and S. Yokoyama. 1997. Selective down-regulation by protein kinase C inhibitors of apolipoprotein-mediated cellular cholesterol efflux in macrophages. *Biochemistry.* **36:** 12045–12052.
33. Yamauchi, Y., C. C. Chang, M. Hayashi, S. Abe-Dohmae, P. C. Reid, T. Y. Chang, and S. Yokoyama. 2004. Intracellular cholesterol mobilization involved in the ABCA1/apolipoprotein-mediated assembly of high density lipoprotein in fibroblasts. *J. Lipid Res.* **45:** 1943–1951.
34. Fielding, P. E., K. Nagao, H. Hakamata, G. Chimini, and C. J. Fielding. 2000. A two-step mechanism for free cholesterol and phospholipid efflux from human vascular cells to apolipoprotein A-1. *Biochemistry.* **39:** 14113–14120.
35. Wang, N., D. L. Silver, C. Thiele, and A. R. Tall. 2001. ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. *J. Biol. Chem.* **276:** 23742–23747.
36. Smith, J. D., W. Le Goff, M. Settle, G. Brubaker, C. Waelde, A. Horwitz, and M. N. Oda. 2004. ABCA1 mediates concurrent cholesterol and phospholipid efflux to apolipoprotein A-I. *J. Lipid Res.* **45:** 635–644.
37. Takahashi, Y., and J. D. Smith. 1999. Cholesterol efflux to apolipoprotein AI involves endocytosis and resecretion in a calcium-dependent pathway. *Proc. Natl. Acad. Sci. USA.* **96:** 11358–11363.

38. Komaba, A., Q. Li, H. Hara, and S. Yokoyama. 1992. Resistance of smooth muscle cells to assembly of high density lipoproteins with extracellular free apolipoproteins and to reduction of intracellularly accumulated cholesterol. *J. Biol. Chem.* **267:** 17560–17566.
39. Barbaras, R., P. Puchois, J. C. Fruchart, and G. Ailhaud. 1987. Cholesterol efflux from cultured adipose cells is mediated by LpAI particles but not by LpAI:AII particles. *Biochem. Biophys. Res. Commun.* **142:** 63–69.
40. Remaley, A. T., B. D. Farsi, A. C. Shirali, J. M. Hoeg, and H. B. Brewer, Jr. 1998. Differential rate of cholesterol efflux from the apical and basolateral membranes of MDCK cells. *J. Lipid Res.* **39:** 1231–1238.
41. Lee, J., A. Shirk, J. F. Oram, S. P. Lee, and R. Kuver. 2002. Polarized cholesterol and phospholipid efflux in cultured gall-bladder epithelial cells: evidence for an ABCA1-mediated pathway. *Biochem. J.* **364:** 475–484.
42. Kiss, R. S., D. C. McManus, V. Franklin, W. L. Tan, A. McKenzie, G. Chimini, and Y. L. Marcel. 2003. The lipidation by hepatocytes of human apolipoprotein A-I occurs by both ABCA1-dependent and -independent pathways. *J. Biol. Chem.* **278:** 10119–10127.
43. Bergeron, J., P. G. Frank, F. Emmanuel, M. Latta, Y. W. Zhao, D. L. Sparks, E. Rassart, P. Denèfle, and Y. L. Marcel. 1997. Characterization of human apolipoprotein A-I expressed in *Escherichia coli*. *Biochim. Biophys. Acta.* **1344:** 139–152.
44. Cohen, J. C., R. S. Kiss, A. Pertsemlidis, Y. L. Marcel, R. McPherson, and H. H. Hobbs. 2004. Multiple rare alleles contribute to low plasma levels of HDL cholesterol. *Science.* **305:** 869–872.
45. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76:** 333–337.
46. Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87:** 206–210.
47. Sakr, S. W., D. L. Williams, G. W. Stoudt, M. C. Phillips, and G. H. Rothblat. 1999. Induction of cellular cholesterol efflux to lipid-free apolipoprotein A-I by cAMP. *Biochim. Biophys. Acta.* **1438:** 85–98.
48. Smith, J. D., M. Miyata, M. Ginsberg, C. Grigaux, E. Shmookler, and A. S. Plump. 1996. Cyclic AMP induces apolipoprotein E binding activity and promotes cholesterol efflux from a macrophage cell line to apolipoprotein acceptors. *J. Biol. Chem.* **271:** 30647–30655.
49. Yeaman, S. J. 2004. Hormone-sensitive lipase—new roles for an old enzyme. *Biochem. J.* **379:** 11–22.
50. Johnson, W. J., S. Y. Jang, and D. W. Bernard. 2000. Hormone sensitive lipase mRNA in both monocyte and macrophage forms of the human THP-1 cell line. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **126:** 543–552.
51. Harte, R. A., L. M. Hulten, H. Lindmark, K. Reue, M. C. Schotz, J. Khoo, and M. E. Rosenfeld. 2000. Low level expression of hormone-sensitive lipase in arterial macrophage-derived foam cells: potential explanation for low rates of cholesteryl ester hydrolysis. *Atherosclerosis.* **149:** 343–350.
52. Stadheim, T. A., G. L. Kucera, M. Barancik, P. Htun, and W. Schaper. 2002. c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for mitoxantrone- and anisomycin-induced apoptosis in HL-60 cells. *Leuk. Res.* **26:** 55–65.
53. Barancik, M., P. Htun, and W. Schaper. 1999. Okadaic acid and anisomycin are protective and stimulate the SAPK/JNK pathway. *J. Cardiovasc. Pharmacol.* **34:** 182–190.
54. Nofer, J. R., R. Feuerborn, B. Levkau, A. Sokoll, U. Seedorf, and G. Assmann. 2003. Involvement of Cdc42 signaling in apoA-I-induced cholesterol efflux. *J. Biol. Chem.* **278:** 53055–53062.
55. Oram, J. F., A. J. Mendez, J. Lymp, T. J. Kavanagh, and C. L. Halbert. 1999. Reduction in apolipoprotein-mediated removal of cellular lipids by immortalization of human fibroblasts and its reversion by cAMP: lack of effect with Tangier disease cells. *J. Lipid Res.* **40:** 1769–1781.
56. Witting, S. R., J. N. Maiorano, and W. S. Davidson. 2003. Ceramide enhances cholesterol efflux to apolipoprotein A-I by increasing the cell surface presence of ATP-binding cassette transporter A1. *J. Biol. Chem.* **278:** 40121–40127.
57. Willaime-Morawek, S., K. Brami-Cherrier, J. Mariani, J. Caboche, and B. Brugg. 2003. C-Jun N-terminal kinases/c-Jun and p38 pathways cooperate in ceramide-induced neuronal apoptosis. *Neuroscience.* **119:** 387–397.
58. Chung, H. S., S. R. Park, E. K. Choi, H. J. Park, R. J. Griffin, C. W. Song, H. Park. 2003. Role of sphingomyelin-MAPKs pathway in heat-induced apoptosis *Exp. Mol. Med.* **35:** 181–188.
59. Vaughan, A. M., and J. F. Oram. 2003. ABCA1 redistributes membrane cholesterol independent of apolipoprotein interactions. *J. Lipid Res.* **44:** 1373–1380.
60. Fielding, P. E., J. S. Russel, T. A. Spencer, H. Hakamata, K. Nagao, and C. J. Fielding. 2002. Sterol efflux to apolipoprotein A-I originates from caveolin-rich microdomains and potentiates PDGF-dependent protein kinase activity. *Biochemistry.* **41:** 4929–4937.
61. Chen, W., Y. Sun, C. Welch, A. Gorelik, A. R. Leventhal, I. Tabas, and A. R. Tall. 2001. Preferential ATP-binding cassette transporter A1-mediated cholesterol efflux from late endosomes/lysosomes. *J. Biol. Chem.* **276:** 43564–43569.
62. Hao, M., S. X. Lin, O. J. Karylowski, D. Wustner, T. E. McGraw, and F. R. Maxfield. 2002. Vesicular and non-vesicular sterol transport in living cells. The endocytic recycling compartment is a major sterol storage organelle. *J. Biol. Chem.* **277:** 609–617.
63. Oram, J. F., and S. Yokoyama. 1996. Apolipoprotein-mediated removal of cellular cholesterol and phospholipids. *J. Lipid Res.* **37:** 2473–2491.
64. Mendez, A. J., J. F. Oram, and E. L. Bierman. 1991. Protein kinase C as a mediator of high density lipoprotein receptor-dependent efflux of intracellular cholesterol. *J. Biol. Chem.* **266:** 10104–10111.
65. Garver, W. S., M. A. Deeg, R. F. Bowen, M. M. Culala, E. L. Bierman, and J. F. Oram. 1997. Phosphoproteins regulated by the interaction of high-density lipoprotein with human skin fibroblasts. *Arterioscler. Thromb. Vasc. Biol.* **17:** 2698–2706.
66. Mendez, A. J., J. F. Oram, and E. L. Bierman. 1991. Role of the protein kinase C signaling pathway in high-density lipoprotein receptor-mediated efflux of intracellular cholesterol. *Trans. Assoc. Am. Physicians.* **104:** 48–53.