

Phosphatidylcholine-mediated aqueous diffusion of cellular cholesterol down-regulates the ABCA1 transporter in human skin fibroblasts

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ABSTRACT

ATP-binding cassette protein A1 (ABCA1) is a cholesterol transporter that contributes to the active transport/removal of excess cellular cholesterol. ABCA1 expression is up-regulated when cells accumulate cholesterol.

Aims: The purpose of this study was to determine any correlation between extracellular phospholipid levels and ABCA1 expression and function.

Methodology: Human foreskin fibroblasts were incubated with cholesterol alone or cholesterol and phosphatidylcholine. Total RNA was isolated and subjected to end-point RT-PCR to compare ABCA1 transcript levels. Cell lysates were subjected to Western blot analysis to compare ABCA1 protein levels. Cells were loaded with radiolabeled cholesterol and cellular cholesterol efflux was measured in the presence and absence of apoE, a cholesterol acceptor. ApoE-dependent efflux was calculated as a measure of ABCA1-mediated efflux.

Results: Here we show that incubation of cholesterol-loaded human skin fibroblasts with L- α -phosphatidylcholine (PC) decreases ABCA1 mRNA and protein levels by 93 % and 57 %, respectively, compared to cells loaded with cholesterol alone. Similarly, PC treatment results in a 25% reduction in ABCG1 mRNA levels compared to cells treated with cholesterol alone, but there is no change in SR-BI transcript levels. Subsequent incubation of phospholipid-treated cells with a cholesterol acceptor such as apoE for 24 hours shows a 65% reduction in ABCA1-mediated cholesterol efflux compared to efflux in cells not treated with PC. During the lipid treatment itself, there is a 2.7-fold greater loss of cholesterol from PC treated cells compared to cells treated with cholesterol alone. Measurement of cholesterol in cellular lipid extracts reveals that cells incubated in the presence of phosphatidylcholine are significantly depleted of cholesterol having only 20% of the cholesterol compared to cells loaded with cholesterol alone.

Conclusion: Thus, phosphatidylcholine facilitates removal of cellular cholesterol, and thereby negating the cholesterol-dependent induction of ABCA1 message, protein, and function.

Keywords: ABCA1, Phosphatidylcholine, Cholesterol transport, Cholesterol efflux, Aqueous diffusion.

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21

22 1. INTRODUCTION

23 The adenosine triphosphate binding cassette protein A1 (ABCA1) is a cholesterol transporter
24 responsible for the removal of free cholesterol and phospholipids from cells. The resulting
25 flow of cholesterol from peripheral tissues to the liver is termed reverse cholesterol transport
26 (RCT), and influences the plasma lipoprotein profile, particularly HDL levels [1,2]. Mutations
27 in the ABCA1 gene are associated with familial hypoalphalipoproteinemia and a higher
28 incidence of cardiovascular disease. The ABCA1-mediated cholesterol transport is a
29 unidirectional efflux path and requires the presence of extracellular cholesterol acceptors
30 such as lipid-poor apolipoprotein (apo) A1 or apo E [2,3]. The lipidation of free apoA-I results
31 in the formation of discoidal pre-beta-migrating HDL particles. The newly acquired
32 cholesterol is esterified by the HDL-associated enzyme lecithin:cholesterol acyltransferase
33 (LCAT) transforming the particle into spherical mature HDL, which carries cholesterol to the
34 liver for degradation [4,5].

35 Other mechanisms have been described for the transport of cholesterol from cells [3,6];
36 these include (i) ABCG1-mediated unidirectional efflux to nascent HDL particles, (ii)
37 bidirectional flux mediated by scavenger receptor class-B type I (SR-BI), and (iii) aqueous
38 diffusion down a concentration gradient. These three pathways can use HDL as a
39 cholesterol acceptor, whereas ABCA1 does not mediate cholesterol efflux to HDL particles.
40 Likewise, ABCG1 cannot use apoA1 or apoE as cholesterol acceptor. Aqueous diffusion and
41 SR-BI-mediated pathways can also efflux cholesterol to phospholipid acceptors. Both
42 processes are bidirectional, with direction of net cholesterol flux determined by the
43 cholesterol concentration gradient. The relative contributions of SR-BI, ABCA1 and ABCG1
44 pathways in cholesterol efflux are not clear, but depend on the relative expression and
45 activity of the three proteins and the phospholipid and apoprotein content of the acceptor
46 particles.

47 ABCA1 mRNA and protein levels are up-regulated by cholesterol-loading [3]. Mendez and
48 colleagues observed an increase in apoA-I-mediated energy-dependent cholesterol efflux,
49 distinct from passive diffusion, from cholesterol-enriched human skin fibroblasts [7].
50 Langmann et al. loaded macrophages with cholesterol by incubation with acetylated LDL
51 and observed an increase in ABCA1 mRNA and protein levels. This effect was erased when
52 cellular cholesterol pools were depleted by incubating the cells with HDL₃ [8]. Rong and

53 coworkers reported transformation of smooth muscle cells into macrophage-like cells upon
54 loading them with cholesterol, observing a 200 % increase in ABCA1 mRNA levels [9].

55 While ABCA1-mediated efflux is a unidirectional gradient-independent process requiring
56 ATP, passive flux of cholesterol via aqueous diffusion is bidirectional, with the net direction
57 of cholesterol movement dependent on the concentration gradient of cholesterol [10,11].
58 Many studies have provided evidence for the exchange of cholesterol between synthetic
59 phospholipid vesicles and the plasma membrane of cells. Slotte and colleagues showed
60 that the exchange of cholesterol between phosphatidylcholine vesicles and rat arterial
61 smooth muscle cells occurs by aqueous diffusion and is enhanced in the presence of bovine
62 serum albumin (BSA) [12]. Bartholow and colleagues examined cholesterol efflux from
63 mouse fibroblasts into the medium containing structurally distinct phospholipids, human
64 serum albumin (HSA), or phospholipid-HSA complexes [13]. Their results indicate that
65 phospholipid-albumin complexes are more efficient acceptors of cholesterol than
66 phospholipids and that the degree of unsaturation, charge, and length of fatty acyl chains of
67 phospholipids affect the efficiency of efflux.

68 Like phospholipid liposomes, spherical lipid-rich HDL particles contain phospholipids at the
69 particle-medium interface and are acceptors of cholesterol via aqueous diffusion. LCAT
70 plays an important role in this process by reducing the amount of free cholesterol present in
71 HDL and thus shifting the equilibrium toward further efflux of cellular cholesterol [14,15].

72 A study by Mendez and colleagues clearly demonstrated the ability of HDL particles to
73 mediate cholesterol efflux via both passive diffusion and an active energy-dependent
74 process [7]. They investigated cholesterol efflux from cultured human skin fibroblasts to a
75 series of acceptors, demonstrating that while apoA-I mediates cholesterol efflux in an
76 energy-dependent manner, phospholipid vesicles and trypsinized HDL, lacking functional
77 apoA-I but having an intact phospholipid monolayer, are acceptors of cholesterol via
78 aqueous diffusion.

79 The goal of our studies was to investigate the effect of phospholipids on the expression of
80 ABCA1 message and protein, and on ABCA1-mediated cholesterol efflux. In view of the fact
81 that ABCA1 expression is regulated by cellular cholesterol content, and that phospholipids
82 facilitate removal of cellular cholesterol by various mechanisms, we hypothesized that
83 phosphatidylcholine may down-regulate ABCA1 expression and function. Our data suggest
84 that phospholipids deplete cellular cholesterol by aqueous diffusion and thereby indirectly
85 down-regulate ABCA1-mediated cholesterol efflux.

86

87 2. METHODS

88 2.1 Cell Culture:

89 Normal human foreskin fibroblasts (FSF) (GM04390) were obtained from the National
 90 Institute of General Medical Sciences Human Genetic Mutant Cell Repository (NIGMS). The
 91 cells were maintained in FSF growth medium (DMEM (Cellgro) supplemented with 10%
 92 FBS, 2mM glutamine, 50units/mL penicillin, 50µg/mL streptomycin, and 10mM HEPES) in a
 93 humidified incubator at 37°C and 5% CO₂. Confluent fibroblasts grown on 12-well plates in
 94 FSF growth medium were maintained in DMEM/BSA (FSF growth medium containing
 95 1mg/ml BSA instead of FBS) and used in all treatments.

96 2.2 RNA Isolation and RT-PCR:

Table 1. Primer sequences for Reverse-Transcription-PCR		
Gene & Amplicon Size (bp)	Primer Type	Primer Sequence
huABCA1 (157)	Forward	5'-AACAGTTTGTGGCCCTTTTG-3'
	Reverse	5'-AGTTCCAGGCTGGGGTACTT-3'
huABCG1 (317)	Forward	5'-GGTTCTTCGTCAGCTTCGAC-3'
	Reverse	5'-GTTTCCTGGCATTCAAGGTGT-3'
huSR-BI (216)	Forward	5'-CTGTGGGTGAGATCATGTGG-3'
	Reverse	5'-GCCAGAAGTCAACCTTGCTC-3'
β-actin (285)	Forward	5'-TCATGAAGTGTGACGTTGACATCCGT-3'
	Reverse	5'-CTTAGAAGCATTTGCGGTGCACGATG-3'

97 RNA was isolated using TRI reagent (Sigma) according to the manufacturer's protocol.
 98 Briefly, the cells were washed twice with cold (4°C) PBS, and harvested in TRI reagent by

99 scraping. The total RNA was isolated using chloroform-isopropanol precipitation. The RNA
100 pellet was dissolved in water and the concentration of RNA was determined using a
101 Spectronic Genesys™ 5 spectrophotometer. Two µg of RNA per reaction was converted to
102 cDNA using random hexamers, dNTPs (0.44mM), and Moloney Murine Leukemia Virus
103 Reverse Transcriptase (M-MLV RT) (all from Promega). The reagents were mixed, and
104 incubated at 25°C for 10 minutes followed by incubation at 42°C for 50 minutes. Second
105 strand synthesis and DNA amplification was accomplished by PCR (25 cycles) using *Taq*
106 polymerase (Promega) using primer pairs shown in Table 1. These indicated amplicon sizes
107 will be obtained only with cDNA and not with any contamination DNA, since the sense and
108 antisense primers are separated by an intron. The PCR reaction mixture contained sense
109 and anti-sense sequence-specific primers (1.9 µM each), dNTPs (380µM), MgCl₂ (1.9mM),
110 *Taq* polymerase (0.19 u/µL), and 1X *Taq* polymerase buffer. The PCR products were
111 resolved on agarose gel (2%) and quantitation of DNA amplicon was accomplished by
112 ImageJ (NIH) analysis.

113 2.3 Western blot analysis of ABCA1 and β-actin:

114 Fibroblasts were chilled, washed twice with cold (4°C) PBS, and scraped into the buffer.
115 The cell were pelleted and solubilized in 50 µL of the cell lysis buffer (50mM Tris-HCl, 2mM
116 CaCl₂, 0.1% Triton X-100) containing the serine protease inhibitors, aprotinin (19 µg/mL),
117 benzamidine (1mM), and PMSF (1mM). The amount of protein in the cell lysates was
118 quantitated by the method of Lowry [16].

119 Equal amounts of protein were resolved under denaturing conditions in a 7.0%
120 polyacrylamide gel and transferred onto polyvinylidene fluoride membrane (Immobilon™-P) in
121 transfer buffer (25mM Tris, 192mM glycine, 15% v/v methanol, pH 8.5) at 30 volts overnight
122 or at 100 volts for 1 hour. The blot was first incubated in blocking buffer (20mM Tris, 150mM
123 NaCl, 2mM CaCl₂, 1% gelatin, 0.05% TWEEN-20, pH 7.4) overnight at room temperature
124 and then probed with anti-ABCA1 rabbit polyclonal antibody (5µg/mL, Novus Biologicals)
125 and peroxidase-conjugated goat-anti-rabbit secondary antibody (Jackson ImmunoResearch
126 Laboratories, Inc.). The Supersignal West Pico chemiluminescent substrate (Pierce) was
127 used to detect the protein bands. The membrane was stripped and reprobed with anti-actin
128 rabbit polyclonal antibody (1.5µg/mL, Santa Cruz Biotechnology) and goat-anti-rabbit
129 secondary antibody. Results of Western Blot analysis were quantitated using the ImageJ
130 software available at the NIH website.

131 2.4 Lipid Treatments:

132 Cholesterol (99+%) was purchased from Sigma and dissolved in absolute ethanol at 20
 133 mg/mL. L- α -phosphatidylcholine from egg yolk, type XIII-E, 100 mg/mL in ethanol was
 134 obtained from Sigma. The lipids were suspended in cell culture media containing 1 mg/mL
 135 BSA and sonicated in a bath sonicator for 8 min to form lipid vesicles. For lipid treatments,
 136 confluent FSF were washed with PBS and incubated in DMEM/BSA containing 20 μ g/mL
 137 cholesterol, and/or 100 μ g/mL PC for 24 hours. **Control wells received vehicle (ethanol)**
 138 **alone.** The cells were harvested in TRI reagent for isolation of total RNA, or in lysis buffer
 139 for protein analysis.

140 2.5 Cholesterol Efflux Assays:

141 **The cholesterol efflux assay was performed as earlier [17].** The experimental protocol is

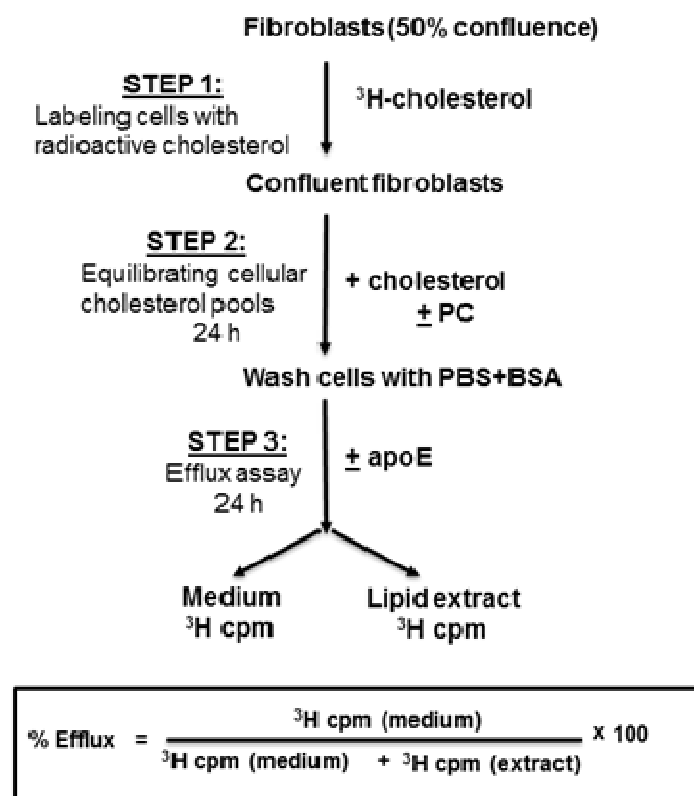


Figure 1. Schematic representation of the cholesterol efflux protocol. Step 1: cells were seeded on 12-well plates and labelled with radioactive cholesterol till they reached confluency. Step 2: The labelled cholesterol was equilibrated throughout the cellular cholesterol pools by incubation for 24 hr with unlabelled cholesterol (20 μ g/mL). For PC treated cells, 100 mg/mL of L- α -phosphatidylcholine was added during this step. The lipids were suspended in BSA containing medium by sonication to ensure that they dispersed into lipid-protein vesicles. Step 3: ABCA1-mediated cholesterol efflux was activated by incubating in the presence of 12.5 μ g/mL of apoE. Control wells without apoE allowed measurement of background ABCA1-independent efflux. The % efflux was calculated using the formula at the bottom.

142 shown schematically in figure 1. Fibroblasts were seeded into 12-well cell culture plates and
143 grown to 50% confluence in FSF growth medium prior to addition of either 1 μ Ci/mL or
144 0.5 μ Ci/mL ([1 α ,2 α (n)-³H]cholesterol (Amersham Biosciences). The cells were allowed to
145 reach confluence, the radioactive medium was aspirated, the cells were washed twice and
146 incubated with 1 mL of DMEM/BSA containing 20 μ g/mL of cholesterol and with or without
147 100 μ g/mL PC. After 24 hr, the medium was aspirated and the cells were incubated in
148 DMEM/BSA containing 0.5mM 8-Br-cAMP with or without 12.5 μ g/mL apolipoprotein E for 24
149 hours. The plates were chilled on ice, the medium was collected into scintillation vials, and
150 the cells were washed 4 times with cold (4 $^{\circ}$ C) HEPES-saline BSA (0.03mM BSA, 138mM
151 NaCl, 5.7mM KCl, 1.8mM CaCl₂, 0.8mM MgSO₄, 3.7mM HEPES, pH 7.4) to remove
152 adherent lipids. A 3:2 hexanes:isopropanol (v/v) solvent was used to extract cellular lipids
153 [18]. Radioactivity was measured using complete counting cocktail (3a70B, Research
154 Products International) and a Beckman, LS 6500 scintillation counter. The amount of the
155 effluxed cholesterol was calculated as: cholesterol efflux (%) = [radioactivity in the medium /
156 (radioactivity in the medium + radioactivity in the lipid extract)] x 100.

157 **2.6 Cholesterol Assay:**

158 Total cellular cholesterol was measured using a cholesterol quantitation kit from Abcam
159 (Cambridge MA). The assay uses cholesterol oxidase to generate hydrogen peroxide which
160 reacts with a sensitive cholesterol probe to generate a colored product (λ_{max} = 570 nm).
161 Cellular lipid extract prepared in 500 μ L/well of 3:2 hexanes: isopropanol. The extract was
162 dried under nitrogen and the dried lipid pellets were resuspended in 50 μ L of the supplied
163 cholesterol assay buffer by vortexing. Samples were treated with cholesterol esterase to
164 hydrolyze any esterified cholesterol to free cholesterol and the resulting total cholesterol was
165 quantified colorimetrically. A standard curve was prepared with cholesterol concentrations
166 ranging from 0.25 μ g to 2 μ g of cholesterol. Results were represented as μ g of cholesterol
167 per sample (i.e. per well of cells). Identically plated and treated wells were assayed for total
168 protein content by the method of Lowry to ascertain equal number of cells in each well.

169 **2.7 Statistical Analysis:**

170 All experiments were repeated at least 3 times. The intensity of DNA and/or protein bands
171 were quantitated using Image J software (NIH). The data were corrected for the
172 housekeeping gene and the percentage change was calculated in treated samples
173 compared to controls. The gel images from one representative experiment are shown with
174 bar graphs (or numeric values) showing averages of 3 or more experiments. For

cholesterol efflux experiments (n=3), standard deviation of the sample was calculated. A Student's t-test, was used to determine statistical significance (P value).

3. RESULTS

3.1 Phosphatidylcholine down-regulates ABCA1 mRNA and Protein levels.

This study aimed to understand the regulation of cholesterol transporters by protein-free phospholipid vesicles. It is already well documented that both ABCA1 and ABCG1 cholesterol transporters are up-regulated by cholesterol loading of cells. SR-BI, which is a bidirectional cholesterol transporter, is not up-regulated by cholesterol. While ABCA1 and ABCG1 are expressed in macrophages and contribute to HDL biogenesis, SR-BI is primarily found on hepatocytes and is involved in the selective uptake of cholesterol esters from HDL particles. We examined the transcription of all three cholesterol transporters after incubation of human foreskin fibroblasts (FSF) with purified L- α -phosphatidylcholine (PC).

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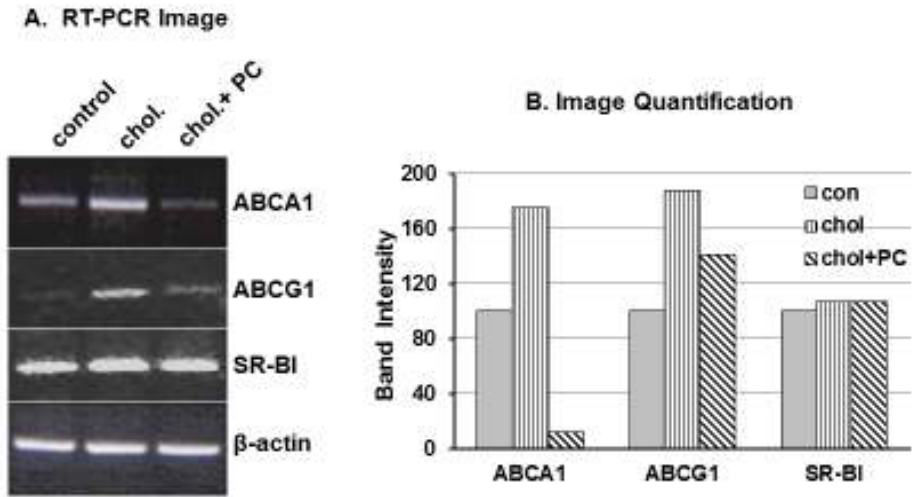
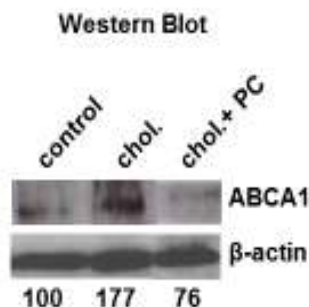


Figure 2. Effect of cholesterol and phosphatidylcholine on the transcription of cholesterol transporters. Fully confluent fibroblasts were incubated for 24 hours in DMEM (BSA) in the absence of lipid (control), the presence of cholesterol (20 µg/ml) alone (chol), or a combination of 20 µg/ml cholesterol and 100µg/mL phosphotidylcholine (chol+PC). Total RNA was extracted, and amplicons for ABCA1, ABCG1, SR-BI and β-actin were amplified by RT-PCR (25 cycles), using gene-specific primers (Table 1). **(A)** PCR products were resolved on 2% agarose gels. **(B)** Data in bar graphs were obtained by quantifying the intensity of bands in gel images using Image J. The transporter to β-actin ratios were calculated and values for control cells was set to 100%. The data are representative of at least three experiments with similar results.

188 As seen in figure 2, loading the cells with 20 µg/mL of cholesterol resulted in a 75% and 88%
 189 increase in the levels of ABCA1 and ABCG1 mRNA levels, respectively. However, when the
 190 culture medium was supplemented with 100 µg/mL of PC in addition to the cholesterol, the
 191 ABCA1 mRNA levels dropped dramatically to only 12% of control levels. Thus, PC negates
 192 the induction of ABCA1 by cholesterol. The presence of PC also down-regulated the
 193 expression of ABCG1 to 75% of the level in cholesterol only treated cells, but did not reduce
 194 it to below the level of control cells. Cholesterol loading or addition of PC did not modulate
 195 the level of SR-BI transcript. Incubation of fibroblasts with varying concentration of PC for 24
 196 hours revealed a dose-dependent effect of the lipid on ABCA1 mRNA levels (data not
 197 shown) with maximum repression seen at 100 µg/mL.

198 Phosphatidylcholine down-regulated ABCA1 protein levels as well. Incubation of fibroblasts
 199 with cholesterol alone resulted in a 77% increase in ABCA1 protein levels as determined by
 200 Western blot analysis (Figure 3). When fibroblasts were treated simultaneously with
 201 cholesterol and PC, the cholesterol-mediated induction of ABCA1 was abolished and ABCA1
 202 protein levels were reduced to 76% of control levels. The intensity of β-actin was used as a
 203 control for cellular protein quantification and gel loading.



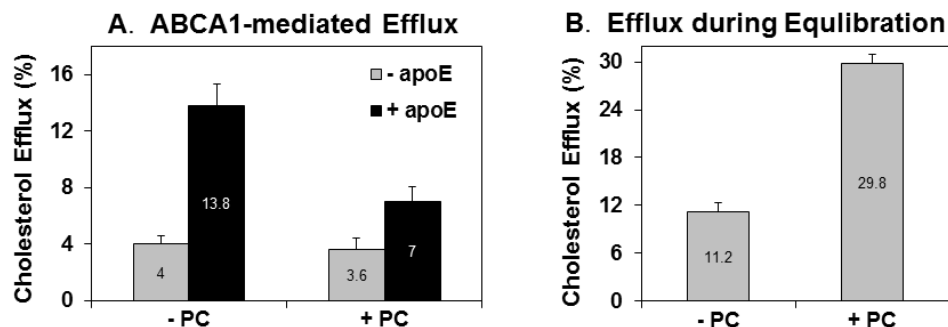
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Figure 3. Phosphatidylcholine negates cholesterol-induced ABCA1 up-regulation. Cells were treated as described in Figure 2 and solubilized in lysis buffer. Cellular protein was quantitated and resolved by 7% SDS-PAGE. Western blot analysis of ABCA1 and β-actin was done as described in Methods. The numbers at the bottom represent relative band intensity as quantified by Image J analysis.

205

206 **3.2 Phosphatidylcholine inhibits ABCA1-mediated cholesterol efflux but** 207 **facilitates aqueous diffusion of cholesterol.**

208 We then asked if a decrease in ABCA1 mRNA and protein levels translates into reduced
209 ABCA1-mediated cholesterol efflux. The efflux protocol is represented in figure 1.
210 Fibroblast monolayers were radiolabeled with [³H]-cholesterol until they reached confluence.
211 This was followed by equilibration of labelled cholesterol into various cellular cholesterol
212 pools by incubating cells with cholesterol alone or in the presence of PC for 24 hours. Next,
213 cholesterol efflux was determined by incubating the cells for 24 hours in lipid-free
214 DMEM/BSA in the presence or absence of apoE. ApoE is a required cholesterol acceptor
215 for ABCA1-dependent efflux pathway, hence the difference in efflux between ± apoE
216 samples was measured as the fraction mediated by ABCA1. As seen in figure 4A, in the
217 absence of PC, the ABCA1 transporter mediated the efflux of 9.8 % of cellular cholesterol.
218 In the presence of PC, ABCA1-mediated cholesterol efflux dropped to just 3.4% of total
219 cellular cholesterol. Thus, the presence of PC resulted in a 65% reduction in ABCA1-
220 dependent cholesterol efflux. The ABCA1-independent diffusion of cholesterol, measured in
221 the absence of apoE, remained unaffected by the presence of PC.



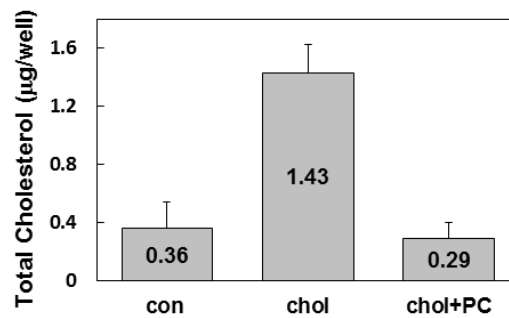
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Figure 4. Phosphatidylcholine down-regulates ABCA1-mediated cholesterol efflux but increases the export of cholesterol by aqueous diffusion. Cholesterol efflux assay was performed as represented in Figure 1. FSF were allowed to reach confluence in FSF growth medium containing tritiated cholesterol. The medium was aspirated, the cells were washed once with 1 ml of HEPES/Saline/BSA, and incubated in DMEM/BSA containing unlabeled cholesterol (20µg/mL) (**chol**) or a mixture of cholesterol (20µg/mL) with phosphatidylcholine (100µg/mL) (**chol + PC**) for 24 hours. **(A)** The cells were then washed 4 times with 1 ml of HEPES-Saline BSA. Subsequently, 1 mL DMEM (BSA) with or without apoE (12.5µg/mL) was added to each well. After 24 hr, the medium was collected, the cells were washed, and lipids were extracted. Radioactivity in the medium and the lipid extract was measured using scintillation counting. Percentage of radioactive cholesterol released was calculated from the formula: % Efflux = $\frac{{}^3\text{H in the medium}}{{}^3\text{H in the medium} + {}^3\text{H in the lipid extract}} \times 100$. Each value is an average of four measurements ± standard deviation. $P(13.8, 7) = 0.0012$. **(B)** Efflux was measured after step 2 of Figure 1. Instead of washing away the lipid treatment (equilibration) medium as in A, it was collected and counted for radioactivity. The cells were washed, and lipids were extracted. The percent cholesterol efflux was calculated as in A. Each value is an average of three measurements ± standard deviation. $P(10.3, 29.8) = 9.1 \times 10^{-7}$.

223

224 3.3 Phosphatidylcholine depletes cellular cholesterol.

225 Thus, our results clearly demonstrate that the presence of PC in the cell culture medium
226 reduces ABCA1 mRNA and protein levels and also reduces ABCA1 function. Furthermore,
227 it negates the up-regulation of ABCA1 induced by cholesterol-loading of cells. Based on
228 these observations, we hypothesized that during the equilibration step, PC either depletes
229 cellular cholesterol content, or prevents cholesterol loading of cells. To test this hypothesis,
230 cell culture medium was saved at the end of the equilibration step and cleared of cellular
231 debris. Cellular lipid extracts were also prepared as above after the equilibration step. Both
232 samples were counted for radioactivity and efflux of radiolabeled cholesterol was calculated.
233 This was used as a measure of ABCA1-independent cholesterol efflux. There was a 2.7-
234 fold increase in the amount of the label released by cells in the presence of PC during 24
235 hours (Fig. 4B). Interestingly, incubation with cholesterol alone did not increase the efflux
236 over control levels (absence of both cholesterol and PC), which was 10.3% + 1.5 (data not
237 shown).



238

Figure 5. PC depletes cellular pool of cholesterol. Total cellular cholesterol content was quantified using a cholesterol quantitation kit from Abcam. Fully confluent fibroblasts were incubated in DMEM/BSA alone (con), or with either 20µg/mL cholesterol (chol) or a mixture of 20µg/mL cholesterol and 100µg/mL PC (chol+PC). The cells were washed 4 times with HEPES-Saline BSA, and lipids were extracted with 3:2 hexanes:isopropanol, dried under nitrogen and resuspended in cholesterol assay buffer. Cholesterol esterase was used to hydrolyze any cholesterol esters in the sample and the resulting total cholesterol was quantified colorimetrically according to the suggested protocol.

239 To further confirm the finding that incubation of fibroblasts with PC depletes cellular
240 cholesterol, we quantified total cellular cholesterol in lipid extracts of cells after the
241 equilibration step. Cells incubated with cholesterol alone showed a 4-fold increase in total
242 cholesterol compared to control cells incubated without any lipid (Figure 5). In the presence

243 of both cholesterol and PC, the cellular cholesterol content dropped to 80% of the level in
244 control cells. In similarly treated separate wells, it was ascertained that the lipid treatments
245 did not alter cell viability and cell number and confluency was comparable between
246 treatments. Results are averages of 4 experiments.

247

248 4. DISCUSSION

249 In our studies, phospholipids consistently reduced ABCA1 mRNA and protein levels as well
250 as ABCA1-mediated cholesterol efflux from fibroblasts. The most likely mechanism by which
251 phospholipids down-regulate ABCA1-mediated cholesterol efflux is the depletion of cellular
252 pools of cholesterol, leading to down-regulation of ABCA1 mRNA and protein levels. This
253 effect can be explained by the cholesterol-accepting capacity of phospholipids. It was
254 previously shown that phospholipid vesicles are efficient acceptors of cellular cholesterol and
255 that the cholesterol-removing capacity of phospholipids increases upon its association with
256 BSA or HSA [13]. In our experiments, both cholesterol and PC were packaged with BSA to
257 form protein-lipid vesicles prior to incubation with fibroblasts.

258 The mechanism of PC-mediated cellular cholesterol depletion is probably by aqueous
259 diffusion. This conclusion is based on elimination of cholesterol transporter mediated
260 pathways for PC-dependent cholesterol export from cells. The efflux did not require a lipid-
261 poor apolipoprotein, which is an absolute requirement of ABCA1-mediated efflux. ABCG1
262 mRNA levels were also reduced in the presence of PC, thus, ABCG1 is probably not
263 responsible for the increased PC-mediated efflux. The remaining transporter, SR-BI,
264 employs phospholipids and HDL particles as cholesterol acceptors, thus it could be a good
265 candidate for facilitating cholesterol removal in the presence of PC. However, there was no
266 change in the level of SR-BI transcript, diminishing the likely-hood for this bidirectional
267 transporter to be responsible for PC-mediated cholesterol export. Aqueous diffusion is also
268 bidirectional and uses phospholipids as an acceptor for cholesterol, making this the most
269 likely mechanism of PC-mediated cholesterol efflux.

270 Aqueous diffusion of cholesterol is driven by a concentration gradient of free cholesterol in
271 the cells and in the culture medium. The molar ratio of PC to cholesterol in the medium was
272 1:4 (100µg/mL PC and 20µg/mL cholesterol) and there is sufficient BSA in the medium, thus,
273 it is expected that the PC-BSA complexes form multi-lamellar vesicles in association with
274 free cholesterol, shifting the equilibrium toward the removal of cellular free cholesterol.
275 Additionally, the presence of PC in the medium sequesters some of the cholesterol and
276 makes it unavailable for cellular uptake. Since cholesterol-enriching of the cells was shown
277 to up-regulate expression, PC-mediated reduction of cholesterol available in the medium,
278 and depletion of cellular cholesterol are expected to produce the opposite effect. This is
279 consistent with the finding that depletion of the cellular pools of cholesterol by HDL and
280 cyclodextrin was shown to reduce ABCA1-mediated cholesterol efflux [8,10,11]. Our data

281 show that phospholipids are also capable of down-regulating ABCA1 expression by a similar
282 mechanism.

283 Quantitative analysis of cellular cholesterol levels supports the model of phospholipid-
284 mediated depletion of cellular cholesterol. The cholesterol content of cells was 5-fold lower in
285 cells incubated in the presence of PC compared to those without. These data confirm that a
286 significant amount of cholesterol was removed from the cells in the presence of
287 phospholipids.

288 Physiologically, phospholipids are most abundant on HDL particles, and HDL particles are
289 potent acceptors of cholesterol via aqueous diffusion and the SR-BI pathways. Our data
290 suggest a role for HDL in regulation of ABCA1-mediated cholesterol efflux *in vivo*. As
291 described earlier, phospholipid- and apolipoprotein-rich HDL particles accept cellular
292 cholesterol via both active lipidation of apoA-I by ABCA1 and passive desorption of
293 cholesterol into the phospholipid monolayer of the particle [2,6]. The results of our studies
294 show that phospholipids of HDL may indirectly deactivate transcription of the ABCA1 gene
295 via depleting cellular cholesterol in the absence of apoA-I. Thus, selective delipidation of
296 plasma, such that neutral lipids are removed, but phospholipids are retained, may result in
297 potent cholesterol acceptors. As suggested by Cham and Chase [19], infusion of such
298 delipidated autologous plasma may be beneficial for the reversal of atherosclerosis.

299

300 **5. CONCLUSION**

301 In conclusion, we showed that phospholipids reduce ABCA1 mRNA and protein levels,
302 decreasing ABCA1-mediated cholesterol efflux. Furthermore, our data suggest that the PC-
303 dependent modulation of ABCA1 occurs via passive efflux of cholesterol into the
304 phospholipid-rich medium and depletion of cellular cholesterol.

305

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309

310 **COMPETING INTERESTS**

311
312 All authors declare that no competing interests exist..
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317 **AUTHORS' CONTRIBUTIONS**

318

319 Author DA performed most of the experiments and wrote the first draft of the manuscript.
320 Author RK also performed some experiments reported in the manuscript. Author JM is the
321 corresponding author who designed the study, supervised the research, and wrote the
322 present version of the manuscript. All authors read and approved the final manuscript.”
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