# cAMP induces ABCA1 phosphorylation activity and promotes cholesterol efflux from fibroblasts

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**Abstract ATP-binding cassette transporter A1 (ABCA1) plays a crucial role in apoA-I lipidation, a key step in reverse cholesterol transport. cAMP induces apoA-I binding activity and promotes cellular cholesterol efflux. We investigated the role of the cAMP/protein kinase A (PKA) dependent pathway in the regulation of cellular cholesterol efflux. Treatment of normal fibroblasts with 8-bromo-cAMP (8-Br-cAMP) increased significantly apoA-I-mediated cholesterol efflux, with specificity for apoA-I, but not for cyclodextrin. Concomitantly, 8-Br-cAMP increased ABCA1 phosphorylation in a time-dependent manner. Maximum phosphorylation was reached in <10 min, representing a 260% increase compared to basal ABCA1 phosphorylation level. Forskolin, a known cAMP regulator, increased both cellular cholesterol efflux and ABCA1 phosphorylation. In contrast, H-89 PKA inhibitor reduced cellular cholesterol efflux by 70% in a dose-dependent manner and inhibited almost completely ABCA1 phosphorylation. To determine whether naturally occurring mutants of ABCA1 may affect its phosphorylation activity, fibroblasts from subjects with familial HDL deficiency (FHD, heterozygous ABCA1 defect) and Tangier disease (TD, homozygous/compound heterozygous ABCA1 defect) were treated with 8-Br-cAMP or forskolin. Cellular cholesterol efflux and ABCA1 phosphorylation were increased in FHD but not in TD cells. Taken together, these findings provide evidence for a link between the cAMP/PKA-dependent pathway, ABCA1 phosphorylation, and apoA-I mediated cellular cholesterol efflux.**—Haidar, B., M. Denis, L. Krimbou, M. Marcil, and J. Genest, Jr. **Cyclic AMP induces ABCA1 phosphorylation activity and promotes cholesterol efflux from fibroblasts.** ***J. Lipid Res.*** **2002.** 43: **2087–2094.**

**Supplementary key words** apolipoprotein A-I • high density lipoprotein • PKA • protein kinase A

The importance of ATP-binding cassette transporter A1 (ABCA1) in reverse cholesterol transport (RCT) process has been strikingly demonstrated by the identification of mutations in ABCA1 gene locus as the molecular defect of Tangier disease (TD) and familial HDL deficiency (FHD) (1, 2). Those patients were characterized by extremely low HDL levels caused by inadequate transport of cellular cholesterol and phospholipids to the extracellular space, leading to hypercatabolism of lipid-poor nascent HDL particles (3). Thus, factors affecting the structure, activity, or concentration of ABCA1 are likely to affect the homeostasis of plasma HDL cholesterol and the RCT process, one of several proposed mechanisms (4) by which HDL may protect against atherosclerosis.

ABCA1 is a 240 kDa protein belonging to a large family of conserved transmembrane proteins that transport a wide variety of substrates, including ions, drugs, peptides, and lipid across cell membranes (5). ABC transporters have been associated with many diseases such as drug-resistant cancer (6), diabetes (7), and cystic fibrosis (8), making these proteins potential targets for therapeutic intervention (9). The ABCA1 gene product is a member of the superfamily of ATP binding cassette transporters predicted to contain 12 transmembrane spanning domains and two nucleotide binding folds (NBF) responsible for the degradation of ATP to provide the energy required for the transport activity (10). The NBF domains contain the highly conserved phosphate-binding loop (11) that forms intimate contacts with the β- and γ-phosphates of bound ATP (12). In some cases, evidence has been provided for a further regulation via phosphorylation of serine/threonine residues in the NBF region (13).

The interaction between apoA-I and ABCA1 is of critical importance for the active apoA-I lipidation, but the structural and functional pathways involved in this process have not been established. Of interest, the earlier attractive concept of Smith et al. (14) suggested that cAMP induces a membrane apolipoprotein receptor that does not lead to endocytosis and degradation, but instead pro-

Abbreviations: ABCA1, ATP-binding cassette transporter A1; CDX, cyclodextrin; CE, cholesteryl ester; CTR, normal control; FC, free cholesterol; FHD, familial HDL deficiency; FRK, forskolin; NBF, nucleotide binding folds; PAGGE, polyacrylamide gradient gel electrophoresis; PKA, protein kinase A; TD, Tangier disease; 8-Br-cAMP, 8-bromo-cAMP.

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*Manuscript received 17 June 2002 and in revised form 2 August 2002.*

*Published, JLR Papers in Press, August 16, 2002.*
*DOI 10.1194/jlr.M200235-JLR200*

motes the transfer of lipids to apolipoprotein. The same group had also reported later that cAMP-mediated cholesterol efflux to apoA-I is associated with the binding, uptake, and resecretion of apoA-I in a calcium-dependent pathway in murine macrophages (15). The effect of cAMP on ABCA1 mRNA and protein appears to be specific to macrophages and is not seen with a number of other cell types (16). Moreover, it has been documented that cAMP induces apoA-I binding activity and promotes cellular cholesterol efflux via ABCA1 protein in macrophages (17). On the other hand, treatment of immortalized fibroblasts with cAMP analog also induces apoA-I-mediated lipid efflux and ABCA1 expression, but these effects are usually modest and require some cholesterol loading of cells (18, 19). We have previously shown that phospholipid breakdown products, mediated by phospholipase C and D, and the activation of protein kinase C modulate cellular cholesterol efflux (20).

The involvement of apoA-I in cellular signaling processes and its property to induce cellular cholesterol efflux via its interaction with ABCA1 led us to hypothesize that cAMP/protein kinase A (PKA)-dependent pathway may modulate cellular cholesterol efflux via activation of ABCA1 by inducing its phosphorylation. The present study aims to provide evidence for links between apoA-I-mediated cellular cholesterol efflux, cAMP, PKA, and ABCA1 phosphorylation, and to examine how these interactions could be affected by cAMP regulators, PKA inhibitors, or naturally occurring mutants of ABCA1 protein.

## METHODS

### Patient selection

Patients with FHD and TD were selected as previously described (1, 2, 21, 22). All these subjects carry a mutation at the ABCA1 gene locus or had clinical signs of TD. For the present study, we selected fibroblasts from two normal control subjects (CTR1-2), five patients with FHD (FHD1–5) and three patients with TD (TD1–3). Cellular studies in all TD subjects revealed a marked impairment of apoA-I-mediated cellular cholesterol efflux. Molecular analysis of the ABCA1 gene revealed a compound heterozygous state for subjects TD1 and TD3 and a homozygous missense point mutation in subject TD2. The protocol for the study was reviewed and accepted by the Research Ethics Board of the McGill University Health Center. Separate consent forms for blood sampling, DNA isolation and skin biopsy were provided.

### Cell culture

Human skin fibroblasts were obtained from 3.0 mm punch biopsies of the forearm of patients and healthy control subjects and were cultured as described (21). We seeded $5 \times 10^4$ cells in 35 mm cell culture wells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.1% nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS. At approximately 50% confluence, 0.2 or 1 μCi/ml [$^3$H]cholesterol was added in the cell medium. When the cells reached confluence, they were washed three times in PBS containing 1 mg/ml of fatty acid free BSA and the medium was replaced by DMEM containing 2 mg/ml BSA and 20 μg/ml of free non lipoprotein cholesterol for 24 h. Cellular cholesterol pools were allowed to equilibrate for another 24 h in DMEM containing 1 mg/ml BSA. Efflux studies (0–24 h) were then performed in DMEM containing 1 mg/ml BSA and 10 μg/ml apoA-I in the presence or absence of specified efflux modulating signaling molecules.

### Cellular cholesterol efflux determination

ApoA-I-mediated cholesterol efflux was carried out on the 10 cell lines; as control, BSA (1 mg/ml) only was used in the efflux medium. All experiments were performed in triplicate and efflux was determined specifically at 24 h. In some experiments, cells were treated with 8-bromo-cAMP (8-Br-cAMP) as described previously (19) with minor modifications. Briefly, the cells received 0.5 mM 8-Br-cAMP during 24 h pretreatment and during efflux time incubation. At the end time, cells were chilled on ice and the medium was collected. To precipitate any floating cells, the medium was centrifuged for 10 min at 2,500 rpm. The cell layer was dissolved overnight in 0.1 N NaOH at 4°C. Cellular protein concentration was determined by the Lowry method (23) and one aliquot was reserved for scintillation counting of cellular [$^3$H]cholesterol content. Cellular cholesterol efflux was determined as follow: $^3$H cpm in medium/($^3$H cpm in medium + $^3$H cpm in cells); the results were expressed as percentage of total radiolabeled cholesterol.

### Cholesteryl ester hydrolysis

The rate of cholesteryl ester hydrolysis was determined by radioisotopic labeling using [$^3$H]cholesterol. At 50% confluence, fibroblasts were radiolabeled with [$^3$H]cholesterol for 48 h then loaded with free cholesterol for an additional 24 h at 37°C as described above. An acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibitor, avasimibe 10 μM (provided by Pfizer Pharmaceutical, Ann Arbor, MI), and 0.5 mM 8-Br-cAMP (Sigma Chemical CO, St. Louis, MO) were added to the equilibration medium supplemented with 1 mg/ml of BSA at 0 h. At subsequent indicated times the cells were washed with PBS and extracted for both lipids and proteins. Cell lipids were extracted with hexane-isopropanol (3:2, v/v) at room temperature for 30 min, followed by a wash with another 0.5 ml of solvent. Protein in the lipid-free wells was extracted with 0.5 ml of 0.1 N NaOH for 30 min at room temperature and measured by Lowry method. Lipid extracts were dried under $N_2$ stream then resuspended in 25 μl of chloroform and plated onto LK6D silica gel 60 TLC plates. Free cholesterol (FC) and cholesteryl ester (CE) were separated from other lipids using heptane-ethyl ether-methanol-acetic acid, 80:30:3:1.5 (v/v/v/v) as the solvent system and visualized with $I_2$. The FC and CE bands were scraped, and radioactivity was measured by liquid scintillation counting.

### ApoA-I preparation

HDL was isolated from fresh plasma of normolipidemic donors using a standard sequential ultracentrifugation with density (1.125–1.210 g/ml) adjusted with KBr. The HDL preparation was delipidated in acetone-ethanol (1:1, v/v) and total proteins were fractionated at 4°C on two Sephacryl S-200 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) columns (2.6 × 100 cm). Fractions contained in the apoA-I peak were extensively dialyzed in 0.01 M $NH_4HCO_3$, then lyophilized and resuspended in PBS at concentration of 1 mg/ml. Protein purity was assayed by polyacrylamide gradient gel electrophoresis (PAGGE) on each apoA-I fraction and appropriate fractions were pooled, dialyzed in PBS, and lyophilized before being stored at −70°C as stock.

### Probes and Northern blots

A 517 bp probe for human ABCA1 and mouse ABCA1 were prepared by reverse transcription performed on total RNA ex-

tracted from normal human skin fibroblast and from J774 mouse macrophages. This was followed by a PCR step using the forward primer 5′-CCTTGGGTTCAGGGGATTAT-3′ and the reverse primer 5′-AGGATTGGCTTCTTCAGGATGTCC-3′. The amplified fragment was subcloned into pGEM-T (Promega, Madison, WI) and used to transform JM109 cells, then sequenced to ensure the right sequence. After digestion of the subcloned product with *Sal*I and *Sac*II, the insert was extracted from agarose gel and $^{32}$P-labeled using the Amersham/Pharmacia Oligolabeling kit. The probe was used at concentration of $10^6$ cpm/ml in Northern blots. Ten to fifteen micrograms/lane of the total cell RNA were loaded on a 1% formaldehyde-containing agarose gel and transferred to a Hybond N+ (Amersham Biosciences, Piscataway, NJ) membrane for probing. Bands were revealed on a Typhoon phosphorimager (Amersham Biosciences, Sunnyvale, CA).

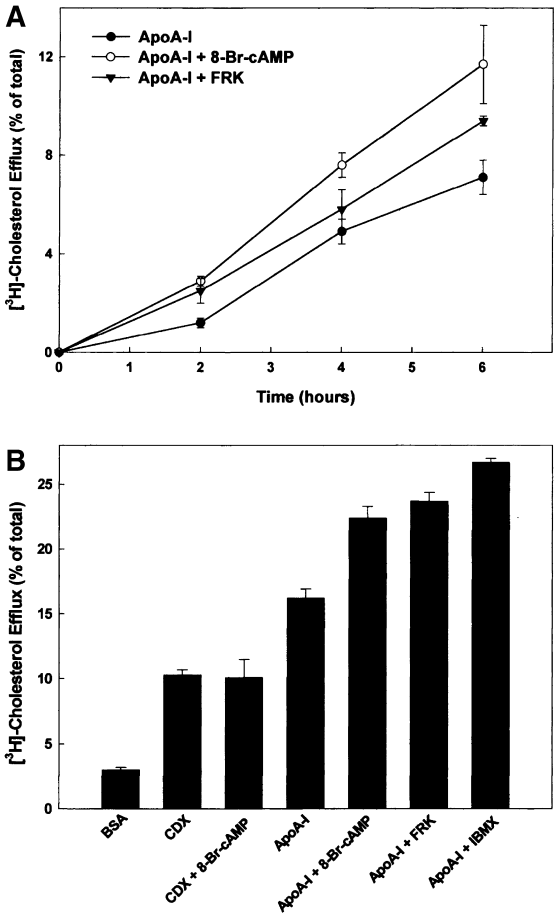


**Fig. 1.** Effect 8-bromo-cAMP (8-Br-cAMP) or cAMP regulator on apoA-I and cyclodextrin-mediated cellular cholesterol efflux. A: Normal control fibroblasts were cholesterol-loaded and [$^3$H]cholesterol-labeled, as described in Methods. Cells were incubated with 10 μg/ml apoA-I alone or in the presence of 0.5 mM 8-Br-cAMP, 5 μM forskolin (FRK), or 1 mM IBMX. After 2, 4, and 6 h incubations, radioactivity in the medium and in the cells was determined. Cholesterol efflux is presented as the percent of total radioactivity recovered from the cells and the medium, and represents the mean ± SD from triplicate wells. B: Normal control fibroblasts were cholesterol-loaded and [$^3$H]cholesterol-labeled. Cells were incubated with 1 mM cyclodextrin (CDX) or 10 μg/ml apoA-I in the presence or absence of 0.5 mM 8-Br-cAMP, 5 μM FRK, and 1mM IBMX. After 24 h incubation, cholesterol efflux was determined as in A. One experiment, representative of three, is shown.

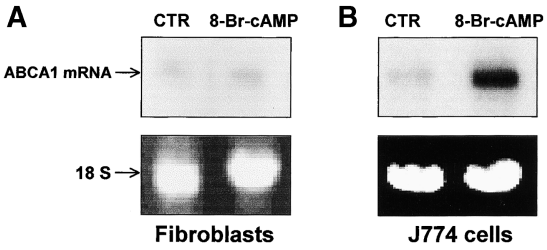


**Fig. 2.** Effect of 8-Br-cAMP on ATP-binding cassette transporter A1 (ABCA1) mRNA expression in normal control fibroblasts and J774 cells. A: Normal fibroblasts were incubated in presence or absence of 1 mM 8-Br-cAMP for 24 h and then harvested. Total RNA was isolated and 15 μg were loaded per lane on agarose gel. After transfer, the blot was probed with human ABCA1 cDNA, as described in Methods. B: J774 cells were incubated in presence or absence of 1 mM 8-Br-cAMP for 24 h and then harvested. Northern blots were incubated with a murine ABCA1 cDNA probe. J744 cells were used as control in this experiment.

## ABCA1 phosphorylation and immunoprecipitation procedures

Confluent fibroblasts were loaded with free cholesterol (20 μg/ml) for 24 h, washed twice in MEM-free phosphate solution (Invitrogen, Burlington, Canada), and incubated with 0.5 mCi/ml of $^{32}$P-labeled orthophosphate for 2 h at 37°C. The cells were washed twice in MEM-free phosphate solution and then treated or not with 1 mM 8-Br-cAMP for varying periods of time as indicated in figures. Cells were also treated with forskolin (FRK) as specified for each experiment. At the indicated time, the cells were washed twice with ice-cold PBS and scraped into 0.5 ml of immunoprecipitation (IP) buffer containing 20 mM Tris (pH 7.5), 0.5 mM EDTA, and 0.5 mM EGTA, 1% Triton-X 100 (Invitrogen, Burlington, Canada) and the suspension was allowed to stand for 30 min at 4°C in presence of a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Cellular debris were removed by centrifugation at 100 *g* for 10 min at 4°C. Protein was estimated by BCA protein assay reagent (Pierce, Canada) according to the protocol from manufacturer using BSA as standard. Cell lysate (150 μg) was cleared of cellular debris by in-

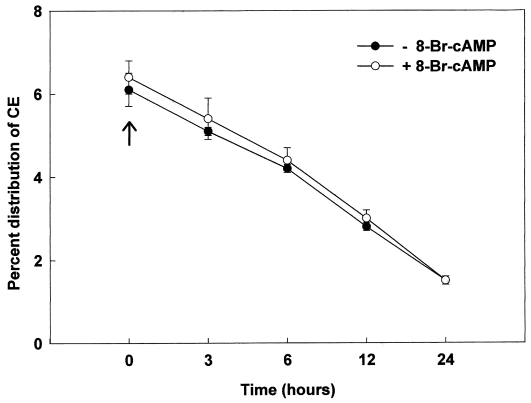


**Fig. 3.** Effect of 8-Br-cAMP on cholesteryl ester (CE) hydrolysis. Normal control fibroblasts were cholesterol-loaded and [$^3$H]cholesterol-labeled, as described in Methods. 0.5 mM 8-Br-cAMP and 10 μM avasimibe, an ACAT inhibitor, were added at 0 h as indicated by vertical arrow in panel. Cellular radiolabeled CE content was determined in each time point by TLC. Results were expressed as percentage of control at 0 h (100%), and represent the mean ± SD from triplicate wells.

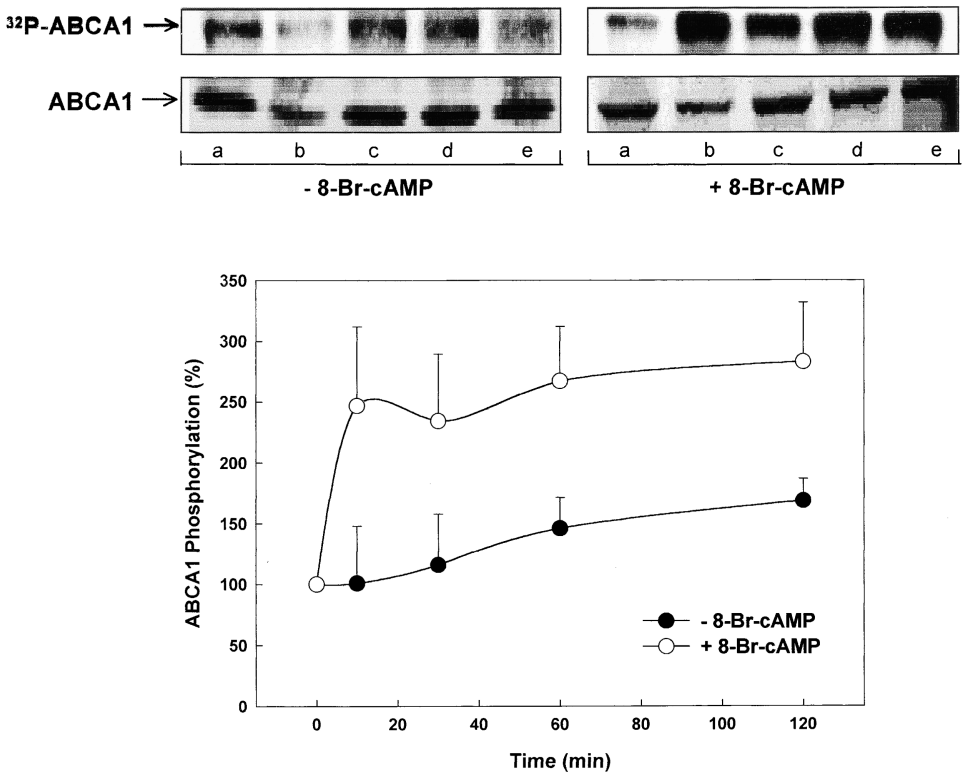


**Fig. 4.** Time-dependent phosphorylation of ABCA1 in the presence or absence of 8-Br-cAMP. Normal intact fibroblasts were incubated with $^{32}$P-labeled orthophosphate for 2 h, and then treated or not with 1 mM 8-Br-cAMP for varying periods of time (0, 10, 30, 60, 120 min; lanes a to e). $^{32}$P-labeled ABCA1 was immunoprecipitated and separated by electrophoresis, and then transferred to PVDF-membrane as described in Methods. $^{32}$P-labeled ABCA1 was revealed and quantified by phosphorimager. Percent increase of $^{32}$P-ABCA1 from three different experiments from the same cells was presented. Plotted values are mean ± SD of triplicate values (lower panel). ABCA1 protein were detected in the same membrane by anti-ABCA1 antibody and used as control for protein loading. $^{32}$P-ABCA1 was not normalized to ABCA1 mass protein. One experiment, representative of three, is shown in the upper panel.

cubation with 20 μl of a 1:1 slurry of protein-A sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and IP buffer for 45 min on a rotating platform at 4°C. Non-specific proteins were separated by centrifugation at 750 *g* for 1 min. The cell lysate was then incubated overnight at 4°C with 3 μg of anti-ABCA1 antibody (AETSDGTLPAP) directed to the intracytoplasmic domain of amino acid residues 1201–1211 of the human ABCA1 gene (Novus Biologicals, Littleton, CO). After addition of slurry of 20 μl protein-A sepharose, the cell lysate was incubated for an additional 2 h. Immunoprecipitates were centrifuged at 750 *g* for 1 min, and washed three times with IP buffer solution, and then boiled for 15 min in a sample buffer containing 0.3 M Tris (pH 6.8), 5% SDS, 50% glycerol, 0.125% bromophenol blue, and 100 mM β-mercaptoethanol. The supernatants were collected by centrifugation at 750 *g* for 2 min. Proteins were separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membrane (PVDF-membrane) (Millipore, Nepean, ON) at 45 V overnight. Bands representing phosphorylated ABCA1 were revealed on a Typhoon phosphorimager (Molecular Dynamics). For protein loading control, the same membranes were rinsed with TBS containing 0.05% Tween 20 (TBST) and blocked with TBST containing non-fat dry milk for 1 h at 25°C. Blots were then incubated overnight at 4°C with primary ABCA1 antibody diluted in TBST (1:100) containing 3% BSA and rinsed with TBST containing 1% dry milk for 5 min at 25°C. Finally, blots were incubated for 1 h at 25°C with donkey anti-rabbit IgG-horseradish peroxidase conjugate (Amersham Biosciences, Piscataway, NJ) diluted in TBST containing 1% dry milk and rinsed with TBST for 5 min. Bands were revealed by enhanced chemiluminescence substrate for horseradish peroxidase (Pierce, Rockford, IL).

## RESULTS

In the present study, we demonstrate that treatment of intact normal fibroblasts with 8-Br-cAMP (0.5 mM) increased apoA-I-mediated cellular efflux by 142%, 55%, and 65% at 2, 4, and 6 h incubation periods, respectively, as shown in **Fig. 1A**. We observed also a significant increase in apoA-I-mediated phospholipid efflux in the presence of 8-Br-cAMP (data not shown). To determine whether this effect of cAMP is not due to passive diffusion of cellular cholesterol, we examined the cellular cholesterol efflux into cyclodextrin (CDX, 1 mM). We found that 8-Br-cAMP did not affect CDX-mediated cholesterol efflux, as shown in Fig. 1B. Control experiments were performed to probe the specificity of apoA-I and 8-Br-cAMP response. We demonstrate that FRK (5 μM), an activator of adenylate cyclase, and IBMX (1 mM), a phosphodiesterase inhibitor known

to be a cAMP regulator, were as effective as 8-Br-cAMP (0.5 mM) in promoting apoA-I-mediated cellular cholesterol efflux in normal cells (Fig. 1A, B). The effect of 8-Br-cAMP on cellular cholesterol efflux was shown to be stable and was essentially unchanged over 24 h. Based on these experiments all cellular cholesterol efflux analysis were made at 24 h.

We tested in normal cells whether the increase in apoA-I-mediated cholesterol efflux induced by 8-Br-cAMP was associated with changes in cellular ABCA1 mRNA. Human skin fibroblasts were incubated for 24 h with 8-Br-cAMP (0.5 mM). There was no effect of cAMP on ABCA1 mRNA levels in fibroblasts (**Fig. 2A**). However, 8-Br-cAMP increased dramatically ABCA1 mRNA in J774 mouse peritoneal macrophages used as control in this experiment (Fig. 2B).

Previous studies showed that cAMP could stimulate neutral cholesteryl ester hydrolase activity in some cell types (24). In order to test the hypothesis that apoA-I-mediated cellular cholesterol efflux stimulation by 8-Br-cAMP was not dependent on an increased CE turnover and free cholesterol availability, normal control fibroblasts were cholesterol-loaded and [$^3$H]cholesterol-labeled, as described in Methods. 0.5 mM 8-Br-cAMP and 10 μM avasimibe, an ACAT inhibitor, were added at 0 h as indicated by vertical arrow (**Fig. 3**). At subsequent times the rate of CE was decreased due to the presence of the ACAT inhibitor. Cellular radiolabeled CE content was determined at each time point by TLC. The addition of 8-Br-cAMP had no effect on the hydrolysis of radiolabeled esterified cholesterol (Fig. 3).

In order to establish the relationship of cAMP/PKA-dependent pathway with ABCA1 phosphorylation activity, ABCA1 was phosphorylated in intact cells using $^{32}$P-orthophosphate, and then immunoprecipitated with anti-ABCA1 antibody, as described in Methods. The specificity of ABCA1-antibody was assessed by immunoprecipitation of [$^{35}$S]methionine labeled-ABCA1, which show a molecular mass of 250 kDa, and was induced by both hydroxycholesterol and 9-*cis*-retinoic acid (data not shown). The phosphorylation of ABCA1 was investigated by treatment of normal cells with 8-Br-cAMP for varying periods of time and untreated cells were used as control for each period of time. Phosphorylated ABCA1 was immunoprecipitated, and then separated by SDS-PAGE (4–12.5%) gel. Gels were transferred to PVDF-ImmobilonP membrane. $^{32}$P-labeled ABCA1 was detected by phosphorimager, and then the same membrane was revealed for ABCA1 protein by anti-ABCA1 antibody, as described in Methods. As shown in **Fig. 4**, treatment of normal intact fibroblasts with 8-Br-cAMP increased ABCA1 phosphorylation in a time-dependent manner. Maximum phosphorylation was reached in less than 10 min, representing a 260% increase compared to basal phosphorylation level of ABCA1 in untreated cells, and remained constant for the remaining 120 min of the experiment. $^{32}$P-labeled ABCA1 was quantified by phosphorimager. Percent increase of $^{32}$P-ABCA1 from three different experiments from the same cell lines is presented (Fig. 4, lower panel). One experiment, representative of three different experiments, is shown in the upper panel of Fig. 4.

In order to provide evidence for a specific role of PKA in apoA-I-mediated cellular cholesterol efflux process and

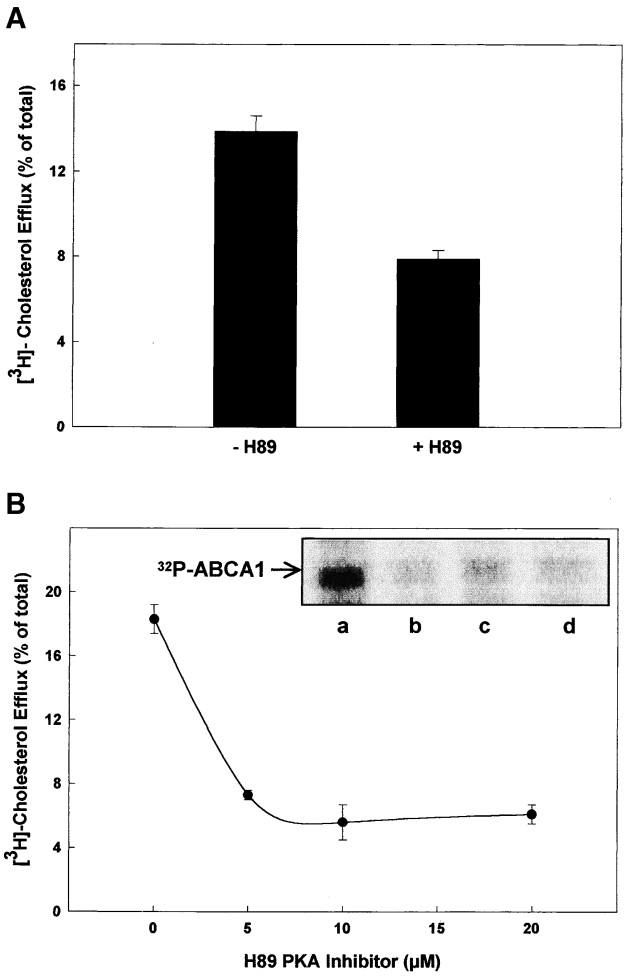


**Fig. 5.** Effect of H-89 protein kinase A (PKA) inhibitor on apoA-I-mediated cholesterol efflux and ABCA1 phosphorylation. A: normal control fibroblasts were cholesterol-loaded and [$^3$H]cholesterol-labeled, as described in Methods. Cells were incubated with 10 μg/m apoA-I in absence or presence of 20 μM H-89. After 24 h incubation, cholesterol efflux was determined. Results represent the mean ± SD from triplicate wells. B: Normal control fibroblasts were cholesterol-loaded and [$^3$H]cholesterol-labeled. Cells were incubated with 10 μg/ml apoA-I in presence of 1 mM 8-Br-cAMP with increasing amounts of H-89 (0, 5,10, 20 μM). After 24 h incubation, cholesterol efflux was determined. Results represent the mean ± SD from triplicate wells. B, inset: normal intact fibroblasts were incubated with $^{32}$P-labeled orthophosphate for 2 h in the presence of 1 mM 8-Br-cAMP and H-89 (0, 5, 10, 20 μM, lanes a to d). $^{32}$P-labled ABCA1 was revealed as described in Fig. 4. One experiment, representative of two, is shown.

ABCA1 phosphorylation activity, we examined the effect of H-89 PKA inhibitor on cellular cholesterol efflux and ABCA1 phosphorylation. Treatment of normal intact cells with 20 μM H-89 decreased significantly basal apoA-I-mediated cholesterol efflux level in the absence of 8-Br-cAMP (**Fig. 5A**) and also basal ABCA1 phosphorylation level (data not shown). In addition, H-89 reduced cellular cholesterol efflux by 70% in a dose-dependent manner in the presence of 1 mM 8-Br-cAMP (Fig. 5B). On the other hand, H-89 inhibits almost completely in a dose dependent manner ABCA1 phosphorylation (0, 5, 10, 20 μM; lanes a to d), as shown in the inset of Fig. 5B. Interestingly,

TABLE 1. Molecular characterization of ABCA1 gene in study subjects

| Cell Lines | HDL-C | Nucleotide Change | Predicted Protein Alteration |
|---|---|---|---|
| | *mmol/l* | | |
| CTR1 | 1.63 | — | — |
| CTR2 | 1.20 | — | — |
| FHD1 | 0.27 | Exon 14 Δ2017-9 | ΔL693 |
| FHD2 | 0.18 | Exon 18 C2665T | R909X |
| FHD3 | 0.39 | Exon 41 Δ5618-23 | ΔED1893,4 |
| FHD4 | 0.18 | Exon 48 C6370T | R2144X |
| FHD5 | 0.09 | Exon 36 GG5277,8C | fs 1628G, Q1636X |
| TD1 | <0.1 | Exon 30 T4369C; Exon 24 splice site G→C | C1477R; Part of the transcript deleted |
| TD2 | <0.1 | Exon 13 A1730G | Q597R |
| TD3 | <0.1 | Exon 48 ΔC6370; nd | 2145X; nd |

FHD1–5 are heterozygous for the reported mutation; TD1,3 are compound heterozygous and TD2 is homozygous. nd, not determined.

we observed that inhibition of the serine/threonine phosphatases by okadaic acid increased the phosphorylation of ABCA1 in normal cells (data not shown).

To determine whether naturally occurring mutants of ABCA1 may affect cellular cholesterol efflux and ABCA1 phosphorylation activity, fibroblasts from subjects with FHD and TD (**Table 1**) were treated with 8-Br-cAMP or FRK. Cellular cholesterol efflux was increased in control, FHD, but not in TD cells (**Fig. 6A**) in response to 8-Br-cAMP treatment. Furthermore, cells from a normal control (CTR1), FHD (FHD4), and TD (TD1) subjects (Table 1) were incubated with $^{32}$P-orthophosphate in the presence or absence of 8-Br-cAMP (1 mM). As shown in Fig. 6B (upper panel), ABCA1 phosphorylation was increased in control and FHD cells compared to basal phosphorylation levels in the absence of 8-Br-cAMP. However, under the same condition, ABCA1 from TD1 cells shows no further phosphorylation over baseline. The control experiment was performed to verify the 8-Br-cAMP response. We demonstrated that FRK (5 μM) was as effective as 8-Br-cAMP (0.5 mM) in inducing ABCA1 phosphorylation in control and FHD, but not in TD cells (Fig. 6B, lower panel).

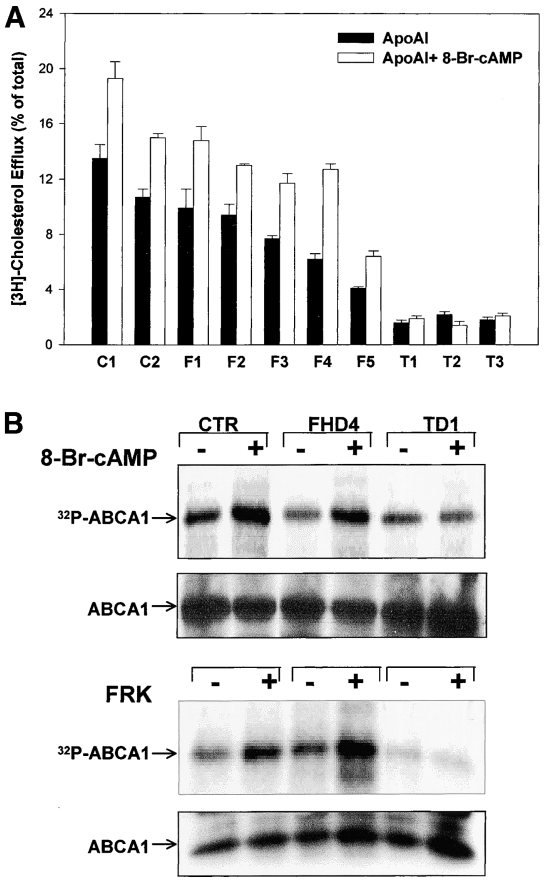


**Fig. 6.** Specific naturally occurring mutations of ABCA1 affect its cellular cholesterol efflux and phosphorylation activities. A: Fibroblasts from normal control, familial HDL deficiency (FHD), and Tangier disease (TD) subjects (Table 1), were cholesterol-loaded and [$^3$H]cholesterol-labeled, as described in Methods. Cells were incubated with 10 μg/ml apoA-I alone or in the presence of 0.5 mM 8-Br-cAMP. After 24 h incubation, radioactivity in the medium and in the cells was determined. Cholesterol efflux is presented as the percent of total radioactivity recovered from the cells and the medium, and represents the mean ± SD from triplicate wells. B, upper panel: Fibroblasts from normal control 1 (CTR1), FHD4, and TD1 subjects (Table 1) were radiolabeled with $^{32}$P-orthophosphate and treated with 1 mM 8-Br-cAMP for 1 h. ABCA1 was identified as described in Fig. 4. Lower panel: Fibroblasts from CTR1, FHD4 and TD1 subjects (Table 1) were radiolabeled with $^{32}$P-orthophosphate and treated with 5 μM FRK for 2 h. ABCA1 was identified as described in Fig. 4. One experiment, representative of two from the same subjects, is shown in upper and lower panels.

## DISCUSSION

cAMP has been recognized to promote apoA-I-mediated cellular lipid efflux (14). Here we have shown that 8-Br-cAMP increased apoA-I-mediated cellular cholesterol efflux in normal cells, but did not affect diffusion-mediated non-specific cholesterol efflux (Fig. 1B). At the same time, treatment of intact normal cells with 8-Br-cAMP increased ABCA1 phosphorylation in a time-dependent manner. At 10 min, a 2.5-fold increase in ABCA1 phosphorylation was observed compared to the basal phosphorylation level in untreated cells (Fig. 4). The effect of 8-Br-cAMP on cellular cholesterol efflux and ABCA1 phosphorylation was not due to increased ABCA1 mRNA and increased protein levels (Fig. 2A). However, previous studies have documented that both ABCA1 mRNA and protein levels are induced by cAMP treatment in macrophages (16, 17). Furthermore, 8-Br-cAMP did not affect the activity of cholesteryl ester hydrolase (Fig. 3). It is more likely that a cAMP-dependent signaling pathway directly induces or activates key proteins involved in the apolipoprotein-mediated lipid removal pathway.

The effects of 8-Br-cAMP on cellular cholesterol efflux and ABCA1 phosphorylation were supported by experiments showing that FRK, known as an activator of adenylate cyclase, induces both increased cellular cholesterol efflux and ABCA1 phosphorylation in normal cells (Fig. 1A, B and Fig. 6B, lower panel). These are consistent with the concept that a cAMP/PKA-dependent pathway may regulate cellular cholesterol efflux. This concept is further

supported by the finding that: *1*) H-89 PKA inhibitor reduces significantly cellular cholesterol efflux (Fig. 5A, B); and *2*) ABCA1 phosphorylation was also reduced by H-89 (Fig. 5, inset). Additional evidence for the link between ABCA1 phosphorylation and cellular cholesterol efflux was provided by the observation that an increase in apoA-I-mediated cholesterol efflux was observed within the first hour of treatment with 8-Br-cAMP (Fig. 1A), concurrent with the kinetic of phosphorylation of ABCA1 in presence of 8-Br-cAMP (Fig. 4). Of interest, it has been demonstrated that apoE induces Akt/protein kinase B phosphorylation in neuronal cells via cAMP/PKA-dependent pathway (25), and apoA-I stimulates human placental lactogen release by activation of MAP kinase (26). One can speculate that ApoA-I may regulate cAMP levels, which was determine by the balance of cellular adenyl cyclase and phosphodiesterase activities (27) that lead to the activation of PKA and subsequent activation of ABCA1 by increasing its phosphorylation. It would be of interest to determine whether apoA-I mutations affecting its hydrophobic and charged residues in helices regulate the cAMP/PKA-dependent pathway and ABCA1 phosphorylation activity.

Although the structural characteristics of ABCA1 activation and its interaction with apoA-I has not yet been determined, the present study shows that fibroblasts from TD patients (TD1, Table 1) were unable to increase apoA-I cholesterol efflux (Fig. 6A) and ABCA1 protein phosphorylation by 8-Br-cAMP or FRK treatment (Fig. 6B, C). However, 8-Br-cAMP or FRK increased both cellular cholesterol efflux and ABCA1 phosphorylation in normal control and FHD cells. It has been demonstrated that apoA-I directly binds to ABCA1, whose expression substantially increased apoA-I binding (28). The binding of apoA-I to fibroblasts from TD (homozygote) patients has been reported to be abnormal (29), but not in FHD patients (21). In the present study, we examined cell lines from a FHD patient (FHD4, Table 1) with heterozygous truncated mutation in exon 48 (C6370T; Arg2144stop) and a TD patient (TD1, Table 1) with compound heterozygocity for a splice site defect in intron 24 and a point mutation in exon 30 (T4369C; Cys1417Arg). Evidence from the study of these patients suggested that specific mutations in ABCA1 could severely affect its interaction with apoA-I molecules (protein-protein interactions) and subsequently its phosphorylation. Alternatively, abnormal folding of ABCA1 in TD cells may affect the serine/threonine phosphorylation sites. It was reported that a mutation of multidrug resistance protein 2 (ABCC2), substitutions (Ala and Cys) of Trp1254, alters substrate specificity and results in loss of methotrexate transport activity of ABCC2, whereas other substitutions (Phe and Tyr) had no effect (30). In addition, phosphorylation of P-glycoprotein, an ABC transporter protein, was proposed to play important role in its drug-efflux activity (31). The physical interaction between apoA-I and ABCA1 is supported by recent works by Fitzgerald et al. (32) suggesting that apoA-I stimulated cholesterol efflux cannot occur without direct interaction between apolipoprotein and critical residues in two extracellular loops of ABCA1 protein.

Cellular cholesterol efflux appears to be an important determinant of plasma HDL-C levels. This is based on the observation that ABCA1 defects lead to a marked deficiency of plasma HDL-C, and cellular cholesterol efflux correlates with plasma HDL-C level (33). The regulation of ABCA1-mediated cellular cholesterol efflux is complex and is regulated both at the transcriptional and post-transcriptional levels in cells. It might be insufficient, therefore, to increase ABCA1 mRNA levels in peripheral cells such as fibroblasts in order to increase cellular cholesterol efflux. Strategies targeting the modulation of both the amount of ABCA1 protein and its activation by phosphorylation have potential as therapeutic interventions aimed at raising HDL-C levels in plasma of patients at increased risk for developing atherosclerotic vascular disease.

The results presented in this study provide evidence supporting that apoA-I interacts with cAMP/PKA-dependent pathway leading to ABCA1 phosphorylation. This process might play in vivo a key functional role in the activation of ABCA1 by increasing its phosphorylation, allowing cholesterol to bind free apoA-I.

Further study of the role of apoA-I in mediating cellular signaling and its interaction with ABCA1 protein may provide new insights into the mechanism of reverse cholesterol transport, plasma factors affecting HDL metabolism, and the therapeutic potential of ABCA1 in preventing or treating atherosclerotic vascular disease.

This work was supported by grant MOP 15042 from the Canadian Institute of Health Research (CIHR) and grant DOP 40845 from the CIHR research and development program. An unrestricted grant was generously provided by Pfizer Pharmaceutical, Ann Arbor, MI, as part of the CIHR-Industry Grant Program. J.G. is the recipient of the McGill-Novartis/CIHR Chair in Medicine at McGill University. We thank D. Devost for technical advice.

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