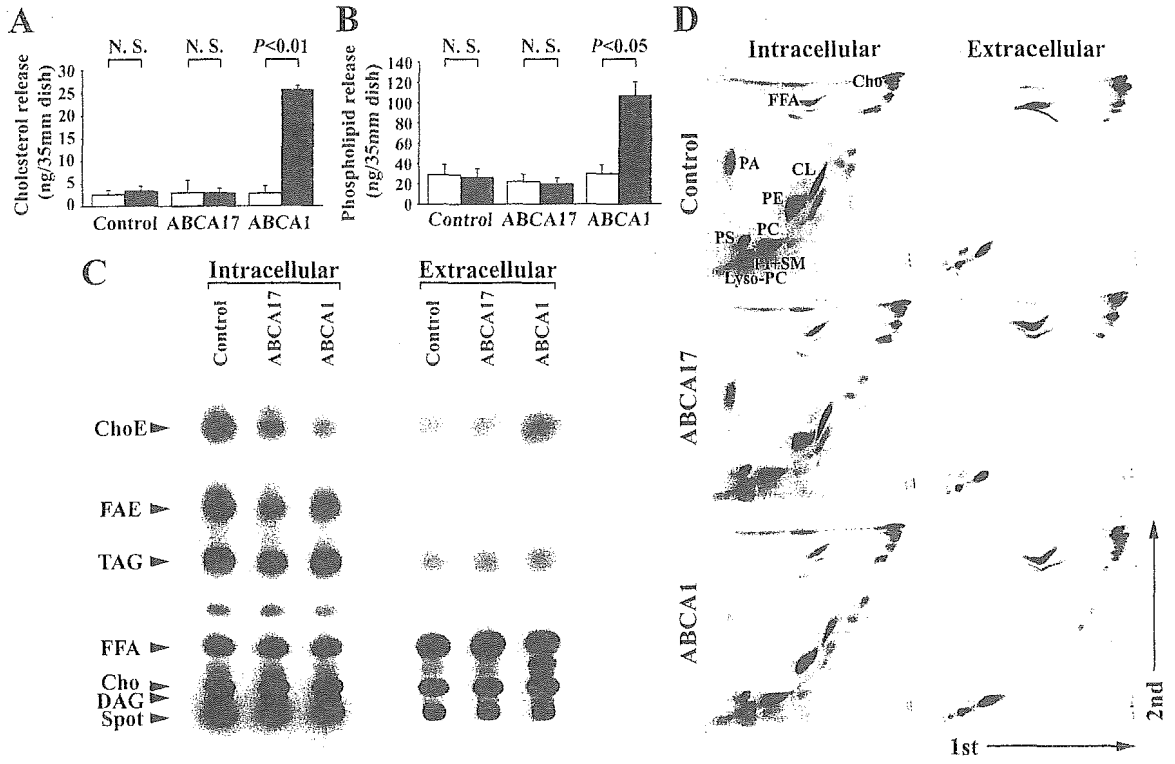

Figure 6 ***Lipid metabolism of HEK293 cells stably expressing ABCA17 or ABCA1***

(**A**, **B**) Release of cholesterol (**A**) and choline-phospholipids (**B**) from ABCA17- and ABCA1-expressing cells and untransfected control cells. Cells were cultured in the absence (open bars) or presence (closed bars) of 10 μg/ml apoA-I and 0.1 % fatty acid-free BSA for 24 h, and released cholesterol and choline-phospholipids were measured. Values represent means $\pm$ S.D. of three independent experiments; N.S., not significant. (**C**, **D**) Metabolic labelling of neutral lipids (**C**) and phospholipids (**D**) of ABCA17- and ABCA1-expressing cells and untransfected control cells. Intracellular ^{14}C-labelled lipids and extracellular ^{14}C-labelled lipids released from cells incubated with [^{14}C]acetate for 72 h were separated by TLC, and representative autoradiographs are shown. Similar results were obtained from at least three independent experiments, and the radioactivity of each of the ^{14}C-labelled lipid species quantified by FLA-5000 is shown in Table 1. ChoE, cholesteryl esters; FAE, fatty acid esters; TAG, triacylglycerols; FFA, non-esterified (free) fatty acids; Cho, cholesterol; DAG, diacylglycerols; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; PS, phosphatidylserine; PA, phosphatidic acid; CL, cardiolipin; Lyso-PC, lyso-phosphatidylcholine.

Table 1 Quantification of metabolically ^{14}C-labelled neutral lipids and phospholipids of ABCA17- and ABCA1-expressing and untransfected control HEK293 cells

*The radioactivity of each of ^{14}C-labelled lipid species, separated by TLC as shown in Figures 6(C) and 6(D), was counted, and values are represented as means $\pm$ S.D. Significance: *$P < 0.05$, **$P < 0.005$ compared with control.* ChoE, cholesteryl esters; FAE, fatty acid esters; TAG, triacylglycerols; NEFA, non-esterified fatty acids; Chol, cholesterol; DAG, diacylglycerols; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; PS, phosphatidylserine; PA, phosphatidic acid; CL, cardiolipin; Lyso-PC, lyso-phosphatidylcholine; *N.D., not detected.*

	Intracellular (d.p.m./1 $\times$ 10^4 cells)			Extracellular (d.p.m./1 $\times$ 10^4 cells)		
Lipids	Control	ABCA17	ABCA1	Control	ABCA17	ABCA1
Neutral lipids	($n = 11$)	($n = 10$)	($n = 4$)	($n = 11$)	($n = 10$)	($n = 4$)
ChoE	14.72 $\pm$ 4.91	3.31 $\pm$ 1.20**	2.06 $\pm$ 0.65**	0.57 $\pm$ 0.25	0.75 $\pm$ 0.41	8.25 $\pm$ 2.15**
FAE	17.72 $\pm$ 6.57	4.69 $\pm$ 1.67**	12.55 $\pm$ 4.37	N.D.[b]	N.D.	N.D.
TAG	17.69 $\pm$ 6.48	10.35 $\pm$ 2.99**	25.11 $\pm$ 6.73	0.91 $\pm$ 0.15	1.17 $\pm$ 0.12**	2.50 $\pm$ 0.37**
NEFA	11.92 $\pm$ 1.89	12.61 $\pm$ 2.29	16.20 $\pm$ 1.79**	30.18 $\pm$ 5.26	43.75 $\pm$ 6.62**	52.81 $\pm$ 13.28**
Chol	57.83 $\pm$ 12.34	70.60 $\pm$ 17.27	112.51 $\pm$ 27.24**	44.68 $\pm$ 6.49	58.08 $\pm$ 5.90**	181.16 $\pm$ 13.15**
DAG	15.69 $\pm$ 5.18	19.51 $\pm$ 7.58	22.30 $\pm$ 4.07*	N.D.	N.D.	N.D.
Phospholipids	($n = 4$)	($n = 4$)	($n = 3$)	($n = 4$)	($n = 4$)	($n = 3$)
PC	394.49 $\pm$ 58.34	354.38 $\pm$ 64.84	375.73 $\pm$ 62.98	12.79 $\pm$ 2.62	12.57 $\pm$ 1.95	41.40 $\pm$ 3.89**
PE	147.38 $\pm$ 20.07	103.48 $\pm$ 24.53*	117.36 $\pm$ 26.44	0.23 $\pm$ 0.07	0.11 $\pm$ 0.03*	0.26 $\pm$ 0.04
PI + SM	70.96 $\pm$ 12.03	65.19 $\pm$ 13.70	91.51 $\pm$ 18.04	4.38 $\pm$ 0.87	3.36 $\pm$ 0.67	11.14 $\pm$ 1.23**
PS	39.15 $\pm$ 4.42	34.66 $\pm$ 7.44	32.98 $\pm$ 8.39	0.53 $\pm$ 0.11	0.21 $\pm$ 0.05**	0.18 $\pm$ 0.02**
PA	6.67 $\pm$ 4.57	6.32 $\pm$ 5.32	1.07 $\pm$ 0.40	N.D.	N.D.	N.D.
CL	34.97 $\pm$ 8.64	23.79 $\pm$ 6.83	12.57 $\pm$ 5.98*	N.D.	N.D.	N.D.
Lyso-PC	3.50 $\pm$ 0.56	4.22 $\pm$ 1.01	3.17 $\pm$ 0.80	0.12 $\pm$ 0.02	0.12 $\pm$ 0.01	0.58 $\pm$ 0.05**

in sperm in the seminiferous tubule of testis. The amino acid identity between mouse ABCA17 and mouse ABCA3 is 55.3 %, and that between mouse ABCA17 and suABCA [32] is 36.7 %. The amino acid identity between suABCA and mouse ABCA3 is 39.7 %, indicating that suABCA has similar identity to ABCA3 as to ABCA17. Since the *ABCA17* gene is at the same locus as the

ABCA3 gene in mouse and rat, the *suABCA* gene could well be a common ancestor of both genes. However, suABCA is expressed in sperm [32], as is ABCA17, while ABCA3 is expressed predominantly in lung alveolar type II cells [26], indicating a function more similar to that of suABCA for ABCA17. Even so, the localization of suABCA and ABCA17 in sperm differs: suABCA is expressed in the entire membrane of sperm in a punctate pattern [32], while ABCA17 is shown in the present study to be expressed at the anterior head of sperm. In addition, the orthologue of ABCA17 has not been isolated in humans.

It is well known that testicular sperm in mammals is morphologically differentiated, yet not able to fertilize the egg, and that sperm acquire the ability to fertilize during resistance in the lumen of the female reproductive tract. This process, capacitation, involves biochemical and physiological changes in the sperm that prepare the cell to undergo the acrosome reaction. Although capacitation occurs in the female reproductive tract *in vivo*, it can be achieved *in vitro*, serum albumin being the essential component of *in vitro* capacitation media [7,33]. Davis et al. [9] found that a decrease in the cholesterol/phospholipid ratio destabilizes sperm membrane, promoting the acrosomal reaction and capacitation. Although the role of albumin in *in vitro* capacitation is not well understood, it is possible that albumin binds lipids, modulating the cholesterol/phospholipid ratio in sperm in a non-specific manner. Go and Wolf [8] reported that the sterol-releasing activity of albumin accounts for this effect in the mouse. However, the mechanism responsible for the decrease in cholesterol in sperm in the physiological situation is unknown.

Recently, the function of ABCA1 and ABCA7 was proposed to be promotion of cellular cholesterol and phospholipid efflux to apoA-I, although the involvement of ABCA7 in cholesterol efflux is controversial [21,22]. In the present study, we investigated the promotion by ABCA17 of cellular lipid efflux to albumin or apolipoproteins, and found that efflux of neither cholesterol nor phospholipid to albumin and apoproteins, including apoA-I, apoE (results not shown) and apoJ (results not shown), was facilitated by ABCA17. Metabolic labelling analysis using [^{14}C]acetate showed that expression of ABCA17 in HEK293 cells reduced the content of intracellular esterified neutral lipids, such as cholesteryl esters, fatty acid esters and triacylglycerols, which suggests that ABCA17 could be involved in capacitation through regulation of the cholesterol/phospholipid ratio in sperm. If this is the case, because capacitation also is observed in human sperm, other ABC transporters, such as ABCA1 and ABCG1, might substitute for ABCA17 in humans, as a functional *ABCA17* gene has not yet been identified in humans or other non-human primates. On the other hand, in mice, ABCA1 mRNA is expressed abundantly in Sertoli cells, but at low levels in isolated germ cells, and ABCA1 mediates lipid efflux from Sertoli cells, which affects male fertility [34]. In additon, ABCG1 mRNA is reported not to be expressed in human testis [35]. We have confirmed in mice by immunohistochemical analysis that ABCA1 and ABCG1 proteins are not expressed in germ cells or sperm, but are expressed mainly in interstitial cells in testis (Supplemental Figure 1; available at http://www.BiochemJ.org/bj/389/bj3890577add.htm). In this context, ABC transporters other than ABCA1 and ABCG1 might be involved in the decrease in cholesterol levels observed in human sperm during capacitation.

The mechanism by which ABCA17 decreases the levels of intracellular esterified neutral lipids is not known. Since ABCA17 is localized on the membrane of the ER when expressed in HEK293 cells, and since esterified neutral lipids such as cholesteryl esters, fatty acid esters and triacylglycerols are synthesized in ER and microsomes [36–40], ABCA17 might be involved in sorting esterified neutral lipids from the ER to other intracellular compartments. On the other hand, ABCA17 expression in HEK293 cells may not reflect its function in sperm, which have a unique structure quite distinct from that of HEK293 cells, and the component in the anterior head of sperm corresponding to the ER is uncertain. Further investigations using sperm are required in order to clarify the physiological role of ABCA17.

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REFERENCES

1 Kopf, G. S., Visconti, P. E. and Galantino-Homer, H. (1999) Capacitation of the mammalian spermatozoon. Adv. Dev. Biochem. **5**, 83–107
2 Davis, B. K. (1981) Timing of fertilization in mammals: Sperm cholesterol/phospholipid ratio as a determinant of the capacitation interval. Proc. Natl. Acad. Sci. U.S.A. **78**, 7560–7564
3 Cross, N. L. (1998) Role of cholesterol in sperm capacitation. Biol. Reprod. **59**, 7–11
4 Visconti, P. E., Westbrook, V. A., Chertihin, O., Demarco, I., Sleight, S. and Diekman, A. B. (2002) Novel signaling pathways involved in sperm acquisition of fertilizing capacity. J. Reprod. Immunol. **53**, 133–150
5 Benoff, S. (1993) Preliminaries to fertilization. The role of cholesterol during capacitation of human spermatozoa. Hum. Reprod. **8**, 2001–2008
6 Travis, A. J. and Kopf, G. S. (2002) The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. J. Clin. Invest. **110**, 731–736
7 Davis, B. K. (1976) Influence of serum albumin on the fertilizing ability *in vitro* of rat spermatozoa. Proc. Soc. Exp. Biol. Med. **151**, 240–243
8 Go, K. J. and Wolf, D. P. (1985) Albumin-mediated changes in sperm sterol content during capacitation. Biol. Reprod. **32**, 145–153
9 Davis, B. K., Byrne, R. and Bedigian, K. (1980) Studies on the mechanism of capacitation: Albumin-mediated changes in plasma membrane lipids during *in vitro* incubation of rat sperm cells. Proc. Natl. Acad. Sci. U.S.A. **77**, 1546–1550
10 Hayden, M. R., Clee, S. M., Brooks-Wilson, A., Genest, Jr, J., Attie, A. and Kastelein, J. J. P. (2000) Cholesterol efflux regulatory protein, Tangier disease and familial high-density lipoprotein deficiency. Curr. Opin. Lipidol. **11**, 117–122
11 Oram, J. F. and Vaughan, A. M. (2000) ABCA1-mediated transport of cellular cholesterol and phospholipids to HDL apolipoproteins. Curr. Opin. Lipidol. **11**, 253–260
12 Schmitz, G. and Langmann, T. (2001) Structure, function and regulation of the ABC1 gene product. Curr. Opin. Lipidol. **12**, 129–140
13 Shulenin, S., Nogee, L. M., Annilo, T., Wert, S. E., Whitsett, J. A. and Dean, M. (2004) *ABCA3* gene mutations in newborns with fatal surfactant deficiency. N. Engl. J. Med. **350**, 1296–1303
14 Allikmets, R. (2000) Simple and complex *ABCR*: genetic predisposition to retinal disease. Am. J. Hum. Genet. **67**, 793–799
15 Lefèvre, C., Audebert, S., Jobard, F., Bouadjar, B., Lakhdar, H., Boughdene-Stambouli, O., Blanchet-Bardon, C., Heilig, R., Foglio, M., Weissenbach, J. et al. (2003) Mutations in the transporter ABCA12 are associated with lamellar ichthyosis type 2. Hum. Mol. Genet. **12**, 2369–2378
16 Orsó, E., Broccardo, C., Kaminski, W. E., Böttcher, A., Liebisch, G., Drobnik, W., Götz, A., Chambenoit, O., Diederich, W., Langmann, T. et al. (2000) Transport of lipids from golgi to plasma membrane is defective in tangier disease patients and *Abc1*-deficient mice. Nat. Genet. **24**, 192–196
17 Weng, J., Mata, N. L., Azarian, S. M., Tzekov, R. T., Birch, D. G. and Travis, G. H. (1999) Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in *abcr* knockout mice. Cell **98**, 13–23
18 Nagata, K., Yamamoto, A., Ban, N., Tanaka, A. R., Matsuo, M., Kioka, N., Inagaki, N. and Ueda, K. (2004) Human ABCA3, a product of a responsible gene for abca3 for fatal surfactant deficiency in newborns, exhibits unique ATP hydrolysis activity and generates intracellular multilamellar vesicles. Biochem. Biophys. Res. Commun. **324**, 262–268
19 Klucken, J., Büchler, C., Orsó, E., Kaminski, W. E., Porsch-Özcürümez, M., Liebisch, G., Kapinsky, M., Diederich, W., Drobnik, W., Dean, M., Allikmets, R. and Schmitz, G. (2000) ABCG1 (ABC8), the human homolog of the *Drosophila white* gene, is a regulator of macrophage cholesterol and phospholipid transport. Proc. Natl. Acad. Sci. U.S.A. **97**, 817–822

20 Kaminski, W. E., Piehler, A., Püllmann, K., Porsch-Özcürümez, M., Duong, C., Bared, G. M., Büchler, C. and Schmitz, G. (2001) Complete coding sequence, promoter region, and genomic structure of the human ABCA2 gene and evidence for sterol-dependent regulation in macrophages. Biochem. Biophys. Res. Commun. **281**, 249–258
21 Wang, N., Lan, D., Gerbod-Giannone, M., Linsel-Nitschke, P., Jehle, A. W., Chen, W., Martinez, L. O. and Tall, A. R. (2003) ATP-binding cassette transporter A7 (ABCA7) binds apolipoprotein A-I and mediates cellular phospholipid but not cholesterol efflux. J. Biol. Chem. **278**, 42906–42912
22 Abe-Dohmae, S., Ikeda, Y., Matsuo, M., Hayashi, M., Okuhira, K., Ueda, K. and Yokoyama, S. (2004) Human ABCA7 supports apolipoprotein-mediated release of cellular cholesterol and phospholipid to generate high density lipoprotein. J. Biol. Chem. **279**, 604–611
23 Dean, M., Hamon, Y. and Chimini, G. (2001) The human ATP-binding cassette (ABC) transporter superfamily. J. Lipid. Res. **42**, 1007–1017
24 Inagaki, N., Gonoi, T., Clement IV, J. P., Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S. and Bryan, J. (1995) Reconstitution of I_{KATP}: an inward rectifier subunit plus the sulfonylurea receptor. Science **270**, 1166–1170
25 Zhao, L.-X., Zhou, C.-J., Tanaka, A., Nakata, M., Hirabayashi, T., Amachi, T., Shioda, S., Ueda, K. and Inagaki, N. (2000) Cloning, characterization and tissue distribution of the rat ATP-binding cassette (ABC) transporter ABC2/ABCA2. Biochem. J. **350**, 865–872
26 Yamano, G., Funahashi, H., Kawanami, O., Zhao, L.-X., Ban, N., Uchida, Y., Morohoshi, T., Ogawa, J., Shioda, S. and Inagaki, N. (2001) ABCA3 is a lamellar body membrane protein in human lung alveolar type II cells. FEBS Lett. **508**, 221–225
27 Tanaka, Y., Yamada, K., Zhou, C.-J., Ban, N., Shioda, S. and Inagaki, N. (2003) Temporal and spatial profiles of ABCA2-expressing oligodendrocytes in the developing rat brain. J. Comp. Neurol. **455**, 353–367
28 Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**, 911–917
29 Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P., Agarwala, R., Ainscough, R., Alexandersson, M., An, P. et al. (2002) Initial sequencing and comparative analysis of the mouse genome. Nature (London) **420**, 520–562
30 Okazaki, Y., Furuno, M., Kasukawa, T., Adachi, J., Bono, H., Kondo, S., Nikaido, I., Osato, N., Saito, R., Suzuki, H. et al. (2002) Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature (London) **420**, 563–573
31 Gibbs, R. A., Weinstock, G. M., Metzker, M. L., Muzny, D. M., Sodergren, E. J., Scherer, S., Scott, G., Steffen, D., Worley, K. C., Burch, P. E. et al. (2004) Genome sequence of the Brown Norway rat yields insights into mammalian evolution. Nature (London) **428**, 493–521
32 Mengerink, K. J. and Vacquier, V. D. (2002) An ATP-binding cassette transporter is a major glycoprotein of sea urchin sperm membranes. J. Biol. Chem. **277**, 40729–40734
33 Yanagimachi, R. (1970) *In vitro* capacitation of golden hamster spermatozoa by homologous and heterologous blood sera. Biol. Reprod. **3**, 147–153
34 Selva, D. M., Hirsch-Reinshagen, V., Burgess, B., Zhou, S., Chan, J., McIsaac, S., Hayden, M. R., Hammond, G. L., Vogl, A. W. and Wellington, C. L. (2004) The ATP-binding cassette transporter 1 mediates lipid efflux from Sertoli cells and influences male fertility. J. Lipid Res. **45**, 1040–1050
35 Chen, H., Rossier, C., Lalioti, M. D., Lynn, A., Chakravarti, A., Perrin, G. and Antonarakis, S. E. (1996) Cloning of the cDNA for a human homologue of the Drosophila white gene and mapping to chromosome 21q22.3. Am. J. Hum. Genet. **59**, 66–75
36 Joyce, C. W., Shelness, G. S., Davis, M. A., Lee, R. G., Skinner, K., Anderson, R. A. and Rudel, L. L. (2000) ACAT1 and ACAT2 membrane topology segregates a serine residue essential for activity to opposite sides of the endoplasmic reticulum membrane. Mol. Biol. Cell **11**, 3675–3687
37 Kaphalia, B. S., Fritz, R. R. and Ansari, G. A. S. (1997) Purification and characterization of rat liver microsomal fatty acid ethyl and 2-chloroethyl ester synthase and their relationship with carboxylesterase (pI 6.1). Chem. Res. Toxicol. **10**, 211–218
38 Kaphalia, B. S. and Ansari, G. A. S. (2001) Purification and characterization of rat hepatic microsomal low molecular weight fatty acid ethyl ester synthase and its relationship to carboxylesterases. J. Biochem. Mol. Toxicol. **15**, 165–171
39 Kaphalia, B. S. and Ansari, G. A. S. (2003) Purification and characterization of rat pancreatic fatty acid ethyl ester synthase and its structural and functional relationship to pancreatic cholesterol esterase. J. Biochem. Mol. Toxicol. **17**, 338–345
40 Owen, M. R., Corstorphine, C. C. and Zammit, V. A. (1997) Overt and latent activities of diacylglycerol acyltransferase in rat liver microsomes: possible roles in very-low-density lipoprotein triacylglycerol secretion. Biochem. J. **323**, 17–21

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Heterogeneity of high density lipoprotein generated by ABCA1 and ABCA7

Michi Hayashi,* Sumiko Abe-Dohmae,* Mitsuyo Okazaki,† Kazumitsu Ueda,§ and Shinji Yokoyama[1,*]

Biochemistry, Cell Biology, and Metabolism,* Nagoya City University Graduate School of Medical Sciences, Kawasumi 1, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan; Laboratory of Chemistry,† College of Liberal Arts and Science, Tokyo Medical and Dental University, Ichikawa 272-0827, Japan; and Division of Applied Life Sciences,§ Graduate School of Agriculture, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan

Abstract The assembly of HDL by helical apolipoprotein and cellular lipid was studied using HEK293 cells to which ecdysone-inducible human ABCA1 or human ABCA7 was transfected. Expression of both ABCA1 and ABCA7 was induced linearly proportional to ponasterone A concentration in the medium. In the experimental conditions used, the ABC protein expression levels limited the rate of lipid release when the apolipoprotein concentration was high, and the apolipoprotein concentration was rate-limiting when the ABC protein expression levels were high. When ABCA1 expression increased in conditions in which it was rate-limiting, relative cholesterol content to phospholipid increased in the HDL produced. In contrast, it was constant when ABCA7 expression increased. To investigate the background mechanism, the HDL particles were analyzed by density gradient ultracentrifugation and high performance lipid chromatography. The ABCA1-mediated reaction produced two distinct HDLs, large cholesterol-rich and small cholesterol-poor particles, and the ABCA7-mediated reaction generated mostly small cholesterol-poor particles. The increase of HDL assembly with the increase of ABCA1 expression was predominant in large cholesterol-rich particles, whereas only small cholesterol-poor HDL increased as ABCA7 expression increased. We conclude that ABCA1 generates cholesterol-rich and cholesterol-poor HDL and that the former is more prominently dependent on the increase of ABCA1 expression. ABCA7 produces this HDL subfraction only as a very minor component.—Hayashi, M., S. Abe-Dohmae, M. Okazaki, K. Ueda, and S. Yokoyama. **Heterogeneity of high density lipoprotein generated by ABCA1 and ABCA7.** *J. Lipid Res.* **2005.** 46: **1703–1711.**

Supplementary key words ATP binding cassette transporter A1 • ATP binding cassette transporter A7 • cholesterol • ecdysone-inducible

ABCA1 mediates the assembly of HDL with extracellular helical apolipoprotein and cellular lipid (1). This reaction is the major source of plasma HDL (2–4) and one of the rate-liming reactions for the regulation of its level (5, 6). The reaction mediates the generation of HDL particles with apolipoprotein, primarily recruiting cellular phospholipid (7). Cholesterol content in these particles is independently regulated by various cellular factors, potentially including protein kinase C and related signaling machineries (8–10), caveolin-1 (11), acyl-CoA:cholesterol acyltransferase (10, 12), and also perhaps other factors relating to intracellular cholesterol trafficking pathways (13). When ABCA1 is transfected to HEK293 cells, which otherwise do not express ABCA1, phospholipid and cholesterol are both released and cholesterol-rich HDL is generated upon incubation of the cells with apolipoprotein A-I (apoA-I) (14–16). ABCA7 also mediates the generation of HDL with apolipoproteins when transfected to HEK293 cells, but the relative cholesterol content to phospholipid in the HDL was lower than that produced by the ABCA1-mediated reaction (16, 17). The relative increase of cholesterol release seemed greater than that of phospholipid when ABCA1 protein level was upregulated by dibutyryl cAMP and phorbol ester (16), so we wondered whether the expression level of ABCA proteins is also a factor that regulates cholesterol content in the HDL. There are many reports that ABCA1 expression can be induced by various factors such as cAMP analogs (18–20), phorbol ester (10, 11), and ligands of liver X receptor or retinoid X receptor, including alteration of cellular cholesterol level (21–24). However, these compounds influence other cellular conditions and sterol metabolism and may not be suitable for examining the isolated effect of the expression level of the ABCA proteins. To address this question,

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Abbreviations: apoA-I, apolipoprotein A-I; DF, 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium; GFP, green fluorescent protein.

[1] To whom correspondence should be addressed.
e-mail: syokoyam@med.nagoya-cu.ac.jp

we used the ecdysone-inducible mammalian expression system (Invitrogen), based on the ability of the insect molting hormone 20-H ecdysone to activate gene expression via the ecdysone receptor (25). Mammalian cells are not responsive to ecdysone or its analog ponasterone A, so the effect of these reagents is minimized other than the expression of a specific gene designed to react. With these cell systems, the expression of ABCA1 and ABCA7 was regulated quantitatively and the generation of HDL was examined accordingly.

Interestingly, the cholesterol content in HDL increased in a dose-dependent manner with ABCA1 but not with ABCA7. The ABCA1-mediated reaction produced large cholesterol-rich and small cholesterol-poor HDL particles, and the former predominantly increased as ABCA1 increased. On the other hand, ABCA7 produced predominantly small cholesterol-poor HDL even when its expression increased.

MATERIALS AND METHODS

Apolipoproteins

ApoA-I and apoA-II were isolated from fresh human HDL (26) and dissolved (19) in phosphate-buffered saline as stock solutions (1 mg/ml) to be stored at 4°C as described elsewhere.

DNA construction and transfection

Full-length cDNAs for human ABCA1 and human ABCA7 were cloned as described previously (15, 16) and subcloned into pIND vector (Invitrogen). ABCA1 cDNA within pEGFP-N1 was digested with *NotI* and ABCA7 cDNA within pEGFP-N1 was digested by *NotI* and *Hind*III. The purified inserts were ligated into pIND to obtain pIND-ABCA1-green fluorescent protein (GFP) and pIND-ABCA7-GFP. HEK293 expressing ecdysone receptor was purchased from Invitrogen and maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DF) supplemented with 10% (v/v) FBS (Hyclone) containing 400 μg/ml Zeocin (Invitrogen). The cloned cDNAs were transfected with Lipofectamine PLUS reagent (Invitrogen) according to the manufacturer's recommendation. Cells permanently introduced were selected with G418, and clones with higher level expression of the fusion proteins were further selected by FACStar (Becton Dickinson).

Evaluation of ABCA1 and ABCA7 expression levels

Total membrane fraction was prepared, and the expression of ABCA1 and ABCA7 was analyzed by immunoblotting using rabbit polyclonal anti-GFP antibody (Molecular Probes) (16).

Cellular lipid-release assay

Cells were seeded onto six-well trays at a density of 1.4×10^6 cells/well with 10% FBS-DF medium and incubated for 24 h. After treatment with ponasterone A of the ABCA1- and ABCA7-transfected cells for 17 and 24 h, respectively, cellular lipid release was induced in the presence of apoA-I or apoA-II (1–20 μg/ml) in DF containing 0.02% BSA and ponasterone A. Lipid was extracted from the medium, and free cholesterol and choline-phospholipid were measured in separate enzymatic assay systems (19).

Density gradient analysis

Cells were subcultured on 100 mm dishes at a density of 8.0×10^6 cells/dish, treated with ponasterone A, and stimulated by apoA-I (2 or 10 μg/ml) for 24 h in 5 ml/dish DF medium containing 0.02% BSA. The media from two dishes were combined and centrifuged to remove cell debris, and 8 ml of the supernatant was processed for sucrose density gradient ultracentrifugation (27). The solution was collected from the bottom of the tube

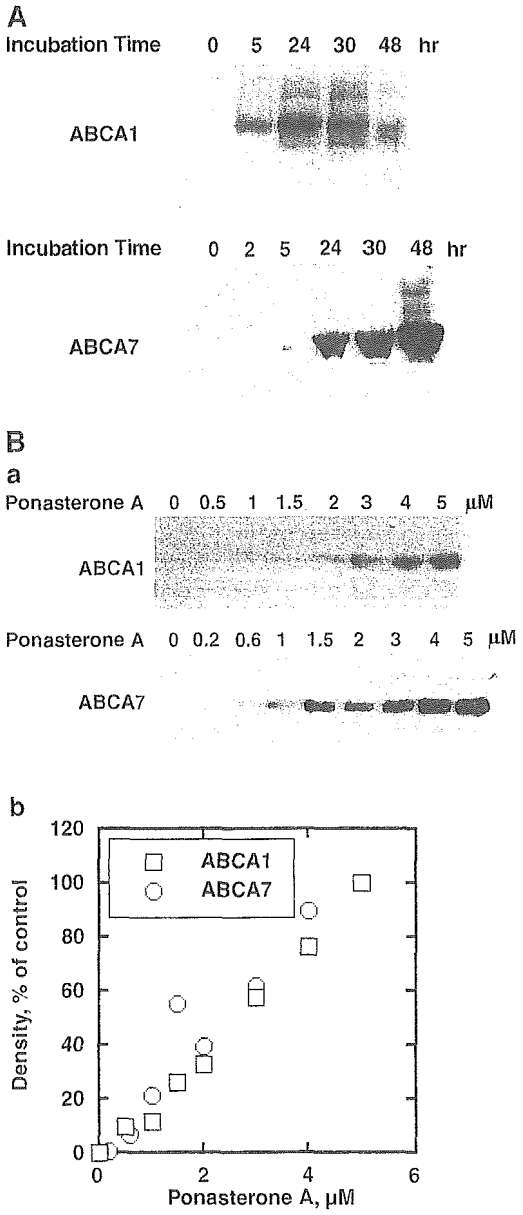

Fig. 1. Induction of green fluorescent protein (GFP)-tagged ABCA1 or ABCA7 expression by ponasterone A. A: The ecdysone-inducible cells were cultured for 24 h and incubated for the indicated times in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 10% FBS in the presence of 5 μM ponasterone A. The membrane fraction (100 μg of protein) was analyzed for ABCA1 or ABCA7 by Western blotting against GFP. B: The ecdysone-inducible cells were cultured for 24 h and incubated for 17 h (ABCA1) or 24 h (ABCA7) in the presence of various concentrations of ponasterone A. The membrane fraction (100 μg of protein) was analyzed for ABCA1 or ABCA7 by Western blotting. The graph represents the density of the bands from the immunoblot quantitated by digital scanning in an Epson GT9500. The results are expressed as percentage of control (5 μM ponasterone A) for ABCA1 and ABCA7 (squares and circles, respectively).

into 13 fractions. The cholesterol and choline-phospholipid contents as well as the density were determined for each fraction (19).

Lipoprotein analysis by HPLC

Lipoproteins in the conditioned medium were analyzed with an HPLC system (28) at Skylight Biotech, Inc. (Akita, Japan). The conditioned medium was centrifuged at 10,000 rpm for 5 min to remove cell debris, and a 200 μl aliquot was applied for an HPLC system using two tandem gel permeation columns (Lipopropak XL; 7.8 mm × 300 mm; Tosoh) with 0.05 M Tris-buffered acetate, pH 8.0, containing 0.3 M sodium acetate, 0.05% sodium azide, and 0.005% Brij-35 at a flow rate of 0.7 ml/min, and an online enzymatic lipid detection system. The method was thoroughly validated against the reference methods of ultracentrifugation and of Superose gel permeation chromatography, including the criteria of subfraction analysis of HDL (29).

RESULTS

Induction of ABCA1 and ABCA7 by ponasterone A

GFP-tagged ABCA1 or ABCA7 cDNA with the ecdysone-inducible mammalian expression system was transfected to HEK293 cells that stably express the ecdysone receptor. It was previously confirmed that attachment of GFP to the C terminus of ABCA1 or ABCA7 did not influence their ability to produce HDL (16, 30). Expression of the ABCA proteins was examined by immunoblotting against GFP. With 5 μM ponasterone A treatment, expression of the GFP-tagged protein became apparent within 5 h, and it increased for 24 h in ABCA1 cells and for 48 h in ABCA7 cells (**Fig. 1A**). Therefore, 24 h induction was chosen as the optimum condition for ponasterone A to induce ABCA1 and ABCA7 for comparison. The dose-dependence of ABCA1 or ABCA7 expression was examined using various concentrations of ponasterone A. The immunoreacting bands of ABCA1-GFP and ABCA7-GFP both linearly increased ponasterone A in a dose-dependent manner (R^2 = 0.99 and 0.94, respectively) (Fig. 1B). At higher concentrations of ponasterone A (>10 μM), the expression levels seemed to reach a maximum (data not shown). The apoA-I-mediated lipid release increased by ponasterone A in a dose-dependent manner (**Fig. 2**). ABCA7 mediated cholesterol release less than ABCA1 with respect to its relative amount of phospholipid release.

Characterization of the lipid release mediated by ABCA proteins and apolipoproteins

Figure 3 demonstrates the release of cholesterol and phospholipid from HEK293 cells by apoA-I in the presence of ABCA1 and ABCA7. Cholesterol was almost all in a free form, and the amount of cholesteryl ester was negligible. When ABC protein expression is high (at high ponasterone A concentrations), apoA-I concentration limits the rate of lipid release. On the other hand, the expression levels of ABCA proteins limit the rate with the ponasterone A concentration used when apoA-I concentration is 10 and 20 μg/ml. **Figure 4** shows the results of similar experiments with apoA-II. The release of cholesterol and phospholipid by apoA-II appeared similar to the results

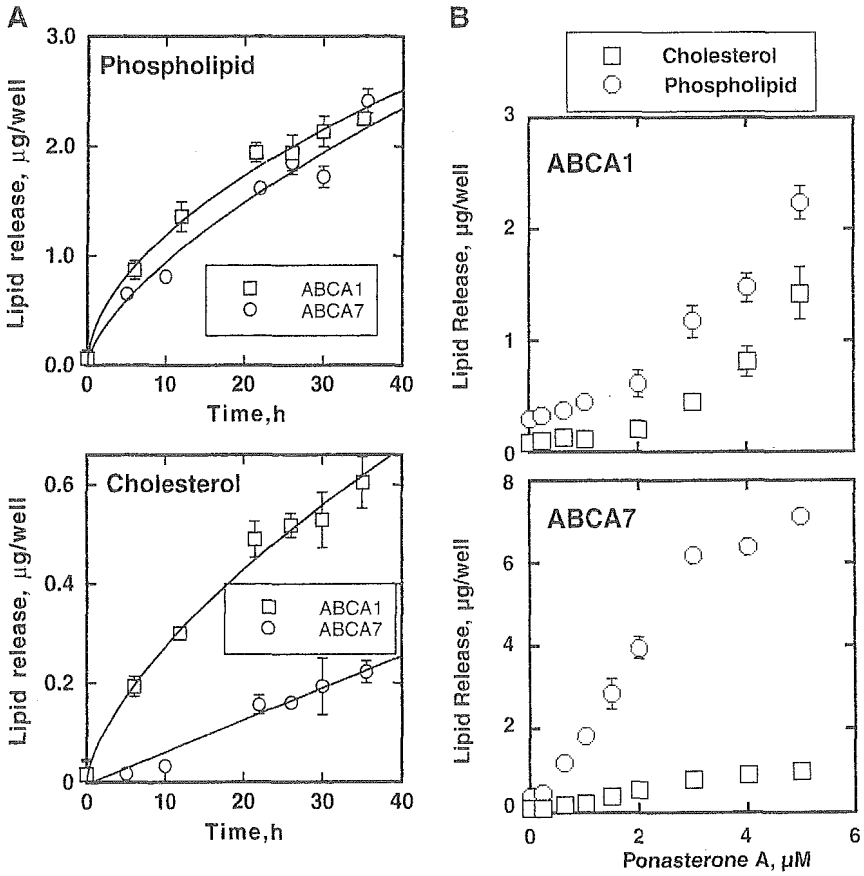

Fig. 2. Apolipoprotein A-I (apoA-I)-mediated release of cholesterol and choline-phospholipid. The ecdysone-inducible cells were incubated with the indicated concentrations of ponasterone A for 17 or 24 h. After washing with buffer H, the cells were incubated with apoA-I. A: Time-dependent profiles of the lipid release at 5 μM ponasterone A. B: Lipid release by apoA-I for 24 h at various concentrations of ponasterone A. Cholesterol (squares) and choline-phospholipid (circles) were determined in the medium. Data represent means ± SD for three samples.

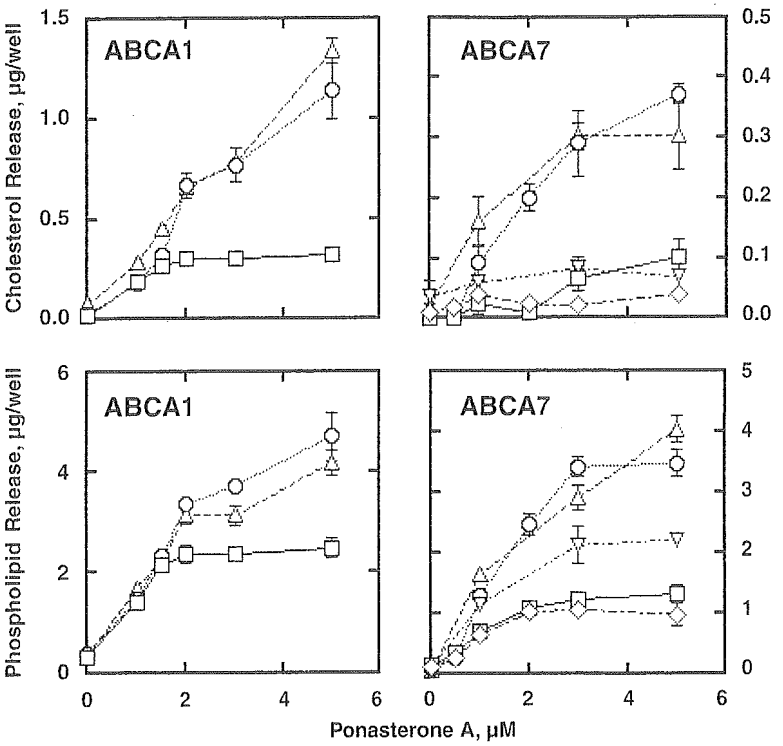

Fig. 3. Lipid release by apoA-I mediated with ABCA1 or ABCA7. The cells were induced for the expression of ABCA1 or ABCA7 in the presence of ponasterone A at 17 and 24 h, respectively. After washing, the cells were incubated with various concentrations of apoA-I [1, 2, 5, 10, and 20 μg/ml (diamonds, squares, inverted triangles, circles, and triangles, respectively)] for 24 h. Cholesterol and choline-phospholipid were measured in the medium. Total cellular cholesterol and choline-phospholipids were 17.5 ± 0.7 and 91.2 ± 2.5 μg/well in the ABCA1-expressing cells and 24.1 ± 2.5 and 116.2 ± 5.8 mg/well in the ABCA7-expressing cells (average ± SD for six samples).

with apoA-I with respect to rate-limiting profiles. When apoA-II concentration is as low as 1 and 2 μg/ml, it limits the rate of lipid release. On the other hand, ABCA1 or ABCA7 limits the rate when apoA-II concentration is as high as 5 and 10 μg/ml.

In the conditions in which ABCA proteins are rate-limiting, the ratio of cholesterol to phospholipid was examined in the released lipid (**Fig. 5**). When the expression of ABCA1 increases by increasing ponasterone A from 1 to 5 μM, the relative content of cholesterol in the released lipid increased by both apoA-I and apoA-II, from 0.1 to 0.4 and from 0.04 to 0.17, respectively (Fig. 5). In contrast, it remained constant at a low level when the expression of ABCA7 increased (Fig. 5).

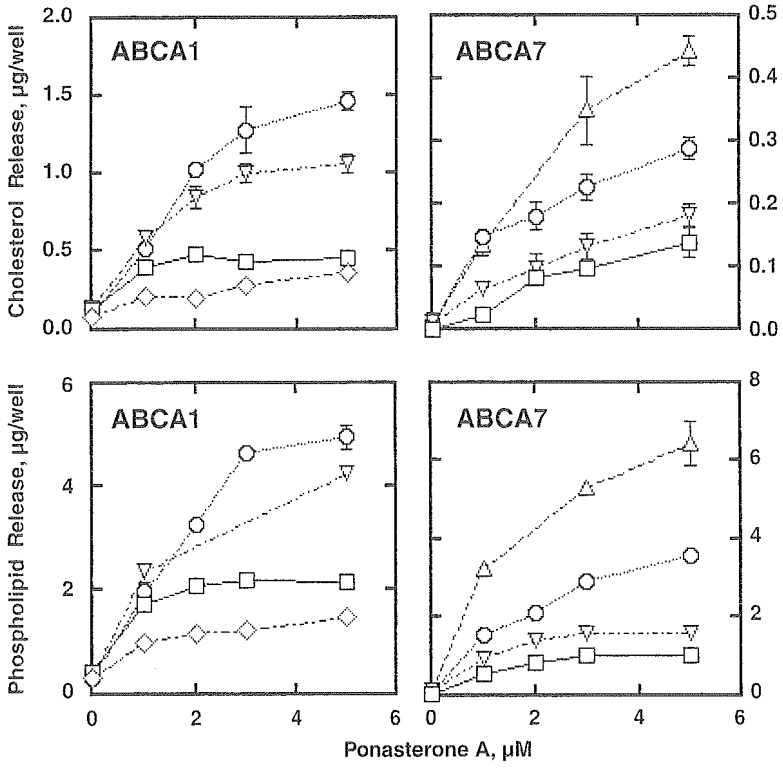

Fig. 4. Lipid release by apoA-II mediated with ABCA1 or ABCA7. The cells were induced for ABCA1 or ABCA7 in the presence of ponasterone A at 17 and 24 h, respectively. After washing, the cells were incubated with apoA-II [1, 2, 5, 10, and 20 μg/ml (diamonds, squares, inverted triangles, circles, and triangles, respectively)] for 24 h. Cholesterol and choline-phospholipid were determined in the medium.

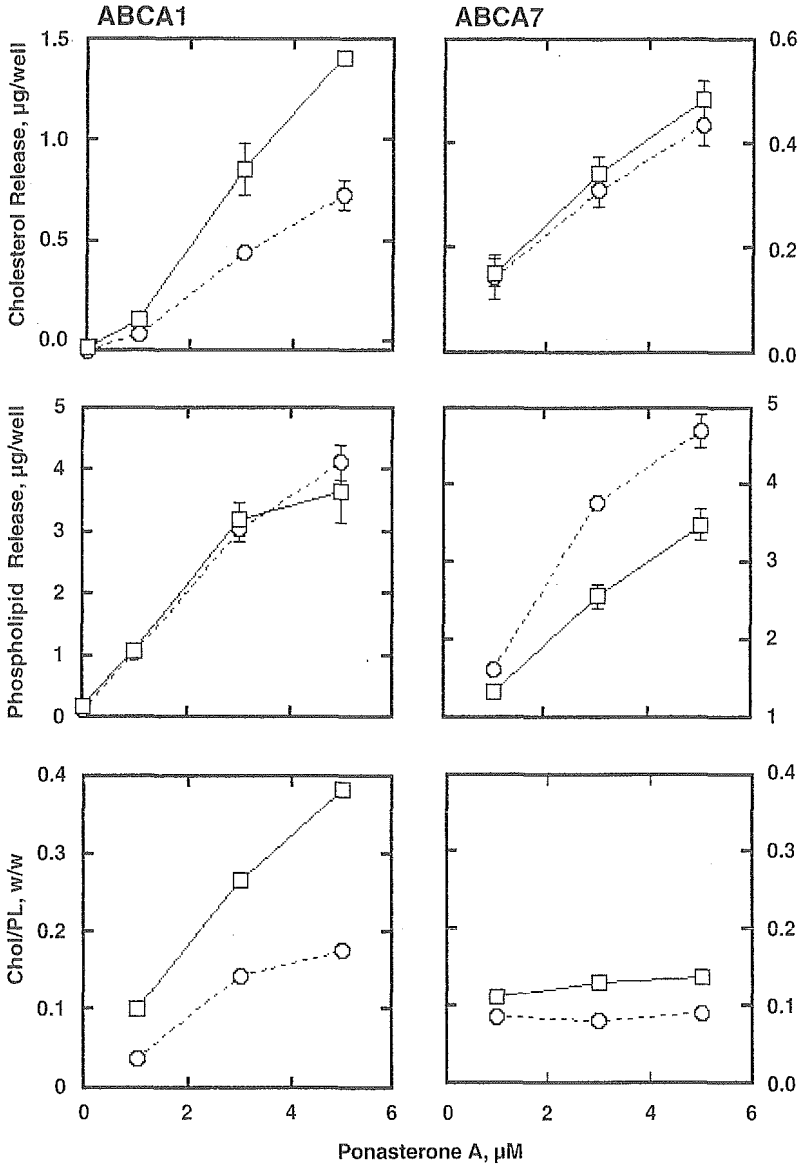

Fig. 5. Cholesterol-phospholipid ratio in the lipid released by apoA-I and apoA-II (squares and circles, respectively) mediated with ABCA1 or ABCA7. Expression of ABCA1 or ABCA7 was induced by ponasterone A, and cellular lipid release was induced by apolipoprotein (10 μg/ml). The ratio of cholesterol to phospholipid (Chol/PL) in mass is shown in the two bottom panels.

Density gradient ultracentrifugation analysis of the conditioned media

The conditioned media with apoA-I of HEK293 cells transfected with ABCA1 or ABCA7 were analyzed by density gradient ultracentrifugation. Both cholesterol and phospholipid were recovered in the fractions, with a density range of HDL to confirm that the lipid was released to form HDL particles. The density of the peak was 1.08 g/ml with the ABCA1-transfected cells and 1.10–1.11 g/ml with the ABCA7-transfected cells (**Fig. 6**), consistent with our previous observation (16). Density profiles of lipid distribution for the ABCA1-transfected cells appear with a shoulder at ~1.11 g/ml, whereas those for the ABCA7-transfected cells were rather symmetric, with a slight irregularity in the lower density side.

Analysis of the media by HPLC

To examine the heterogeneity of the HDL generated, the conditioned media were analyzed by HPLC with a gel permeation column. Elution profiles of cholesterol and phospholipid were monitored online. **Figures 7** and 8 show the results with the apoA-I-conditioned media of the ABCA1- and ABCA7-transfected cells. The medium of the ABCA1-transfeted cells contained two peaks: large cholesterol-rich particles with a diameter of 13 nm, and small cholesterol-poor particles with a diameter of 10 nm. In contrast, the ABCA7-transfected cells generated a predominant peak that is small and cholesterol-poor, accompanied by large particles as a very minor component. These profiles were essentially the same in the conditioned media of HEK293 cells to which ABCA1 or ABCA7 was transiently expressed without the GFP tag (data not shown). When the lipid release was increased either by increasing ABCA1 expression (Fig. 7) or by increasing apoA-I (Fig. 8), a more prominent increase was induced in the large cholesterol-rich particles, and accordingly, the relative amount of cholesterol to phospholipid in the media increased.

In contrast, small cholesterol-poor particles were still major components of the HDL generated by the apoA-I-ABCA7-mediated reaction. This profile did not change even when ABCA7 expression was increased, resulting in

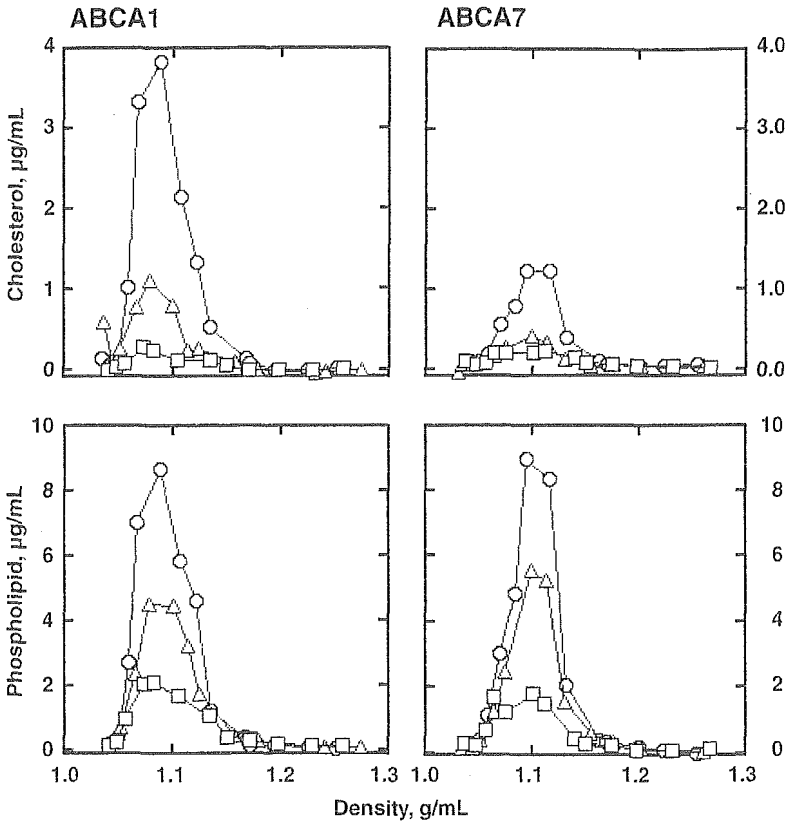

Fig. 6. Density gradient analysis of the lipids released by ABCA1- or ABCA7-expressing cells. The cells were induced for the expression of ABCA1 or ABCA7 by incubating with 5 mM ponasterone A for 17 and 24 h, respectively, and cellular lipid was released by apoA-I [2, 4, and 10 μg/ml (squares, triangles, and circles, respectively)] for 24 h. The medium was analyzed by density gradient ultracentrifugation as described in *Materials and Methods*. Each fraction was determined for density and concentration of cholesterol and phospholipid.

the relative cholesterol content remaining constantly low (Fig. 7). The same tendency was observed when apoA-I was rate-limiting and increased the generation of HDL from the ABCA7-transfeted cells (Fig. 8).

Component analysis of the peaks (31) estimated that the large HDL particles generated by the ABCA1-apoA-I reaction contained cholesterol with 35–38% weight of phospholipid and that the small HDL particles contained

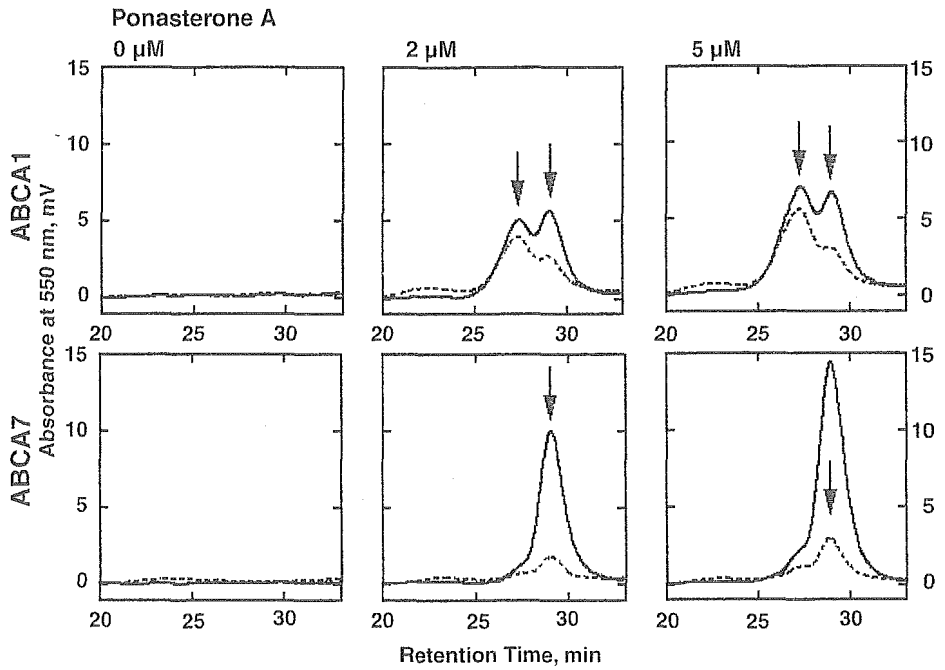

Fig. 7. *HPLC analysis of the lipid released by ABCA1- or ABCA7-expressing cells. Lipoprotein analysis of* the culture medium of ABCA1- or ABCA7-induced HEK293 cells by HPLC. The cells were induced for the expression of ABCA1 or ABCA7 by incubating with ponasterone A (2 and 5 μM) for 17 and 24 h, respectively, and cellular lipid was released by apoA-I (10 μg/ml) for 24 h. The conditioned medium (200 μl) was analyzed by the HPLC lipoprotein analysis system by monitoring cholesterol (broken lines) and phospholipid (solid lines). The arrows indicate eluting positions of the particles with diameters of 13 and 10 nm.

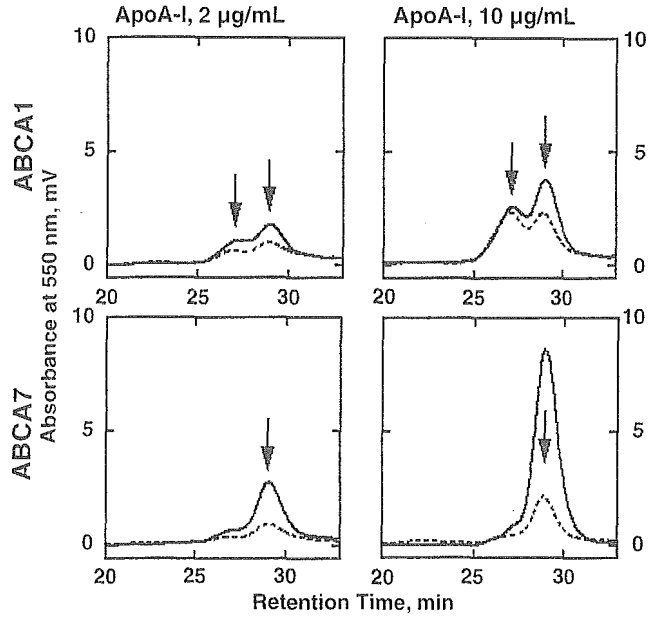

Fig. 8. HPLC analysis of the lipid released by ABCA1- or ABCA7-expressing cells. The cells were induced for the expression of ABCA1 or ABCA7 by incubating with ponasterone A (5 μM) for 17 and 24 h, respectively, and cellular lipid was released by apoA-I (2 and 10 μg/ml) for 24 h. The conditioned medium (100 μl) was analyzed by the HPLC lipoprotein analysis system by monitoring cholesterol (broken lines) and phospholipid (solid lines). The arrows indicate eluting positions of the particles with diameters of 13 and 10 nm.

10–13%. A major HDL component generated by the ABCA7-apoA-I reaction contained 8–11%.

DISCUSSION

ABCA1 is a key cellular factor for the generation of plasma HDL (2–6). ABCA7 was also demonstrated to mediate a similar reaction to ABCA1 to generate HDL in vitro (16, 17). There are several differences between the ABCA1- and ABCA7-mediated reactions with respect to cholesterol content in the HDL generated and posttranscriptional regulation. Recent studies indicated that ABCA7 does not play a major role in lipid release from macrophages (32, 33). However, it may contribute to a source of plasma HDL, at least in female mice (33), and it may be involved in lipid metabolism in kidneys (32) and adipose tissues (33).

The levels of expression of ABCA proteins may also affect these reactions, which could have significant influence on the nature of the products, such as their cholesterol content. However, the regulation of expression of ABCA proteins is multifactorial, including the liver X receptor/retinoid X receptor system (21–24), cAMP (18–20), the calcium-signaling pathway (34), and the peroxisome proliferator-activated receptor α-related system (35, 36). The content of cholesterol in the product HDL is regulated somewhat independently of the HDL assembly reaction itself, potentially with the involvement of such factors as caveolin-1 (11), protein kinase C-related signals (8–10), and intracellular cholesterol level and its esterification (10, 12). To characterize the HDL assembly reaction by ABCA proteins, it is thus important to control the expression level of these proteins without influencing other cellular factors.

We established the HEK293 cell systems with ecdysone-inducible ABCA1 and ABCA7. Both proteins are tagged with GFP, which was shown previously not to interfere with the function of either protein and enabled us to estimate the protein expression levels on a common immunoreactivity basis.

The results of the experiments are summarized below. Expression of ABCA1 and ABCA7 were both linearly proportional to the concentration of ponasterone A in the culture medium. The release of cellular cholesterol and phospholipid is limited by both ABCA protein expression levels and extracellular apolipoprotein levels. When ABCA proteins are rate-limiting, cholesterol release relative to phospholipid increased as ABCA1 expression increased, whereas it remained constant when ABCA7 expression increased. Increase of ABCA1 expression and apolipoprotein concentration both resulted in the increase of cholesterol content in HDL, and it was attributed to expansion of production of this component. On the other hand, ABCA7 mediated the generation of HDL particles that are predominantly small and cholesterol-poor. Thus, ABCA1 is more effective in releasing cellular cholesterol than is ABCA7 by producing large cholesterol-rich HDL. Both ABCA1 expression and apolipoprotein concentration increased relative to the release of cholesterol to phospholipid, and this was attributable to the expansion of production of this component.

Heterogeneity in nascent HDL particles produced by cell-apolipoprotein interaction has been reported in a previous study. When CHO-C19 cells were incubated with 8 μg/ml apoA-I for 24 h, 9.0 and 11.2 nm particles were produced (37). In another experiment with J774 macrophages, incubation with apoA-I led to the formation of more than one type of apoA-I-containing lipid particles, 9 and 12 nm in diameter, having cholesterol-phospholipid ratios of 1:1 to 1:3 (mol/mol), respectively (38). We also reported density profiles of HDL generated by peritoneal macrophages and apoA-I, which suggested similar heterogeneity of the HDL particles (8). The physicochemical and molecular bases for the size heterogeneity of discoidal HDL can be discussed (39), but it is not clear how such a mechanism can be applied when HDL is organized upon the apolipoprotein interaction with ABCA1 and membrane lipid. Lipid composition and particle size are related to the structure of specific domains of apoA-I on HDL (40). ABCA1 may induce specific modification of apoA-I conformation, either directly or indirectly, and such a change may cause parallel changes in its ability to bind to phospholipid bilayers and to the integration of cholesterol (41).

A higher expression level of ABCA1 causes the increase of large cholesterol-rich HDL particles. Although the exact mechanism by which ABCA1 functions in the membrane is not known, a few interesting findings have been

reported. ABCA1 is said to alter the microenvironment of the plasma membrane and influence lipid-protein complex formation in the membrane (42). It forms an oligomeric structure and is related to its function for the biogenesis of nascent apoA-I-containing HDL (43). If oligomeric ABCA1 produces cholesterol-rich HDL, it is consistent with the view that a higher expression of ABCA1 and therefore an increase of oligomeric ABCA1 in the membrane may cause more production of cholesterol-rich particles. The apparent biphasic increase of the lipid release by the increase of ABCA1 expression (Fig. 2B) may also be consistent with this view. ABCA7 may not act in such a manner, and production of cholesterol-poor HDL simply increased when its expression increased. Tall and colleagues (32) suggested that the small amount of cholesterol release by the ABCA7-mediated reaction is attributable to its nonspecific acquisition by the phospholipid-HDL. It is not clear whether the small HDLs demonstrated in the present study gain cholesterol only by such a mechanism.

We have demonstrated a fundamental difference between ABCA1-mediated and ABCA7-mediated HDL assembly. ABCA1 mediates two types of HDL particles, large cholesterol-rich and small cholesterol-poor particles, and the generation of large cholesterol-rich particles is perhaps responsible for the efficient release of cell cholesterol. ABCA7 mediates the generation of only the latter type of particle. At higher expression, ABCA1 produces predominantly more large cholesterol-rich particles, whereas ABCA7 increases the production of only small cholesterol-poor HDL. The results presented here provide a new biochemical basis for understanding an HDL assembly pathway that involves ABCA1 or ABCA7.

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REFERENCES

1. Yokoyama, S. 2000. Release of cellular cholesterol: molecular mechanism for cholesterol homeostasis in cells and in the body. *Biochim. Biophys. Acta.* **1529:** 231–244.
2. Bodzioch, M., E. Orso, J. Klucken, T. Langmann, A. Bottcher, W. Diederrich, W. Drobnic, S. Barlage, C. Buchler, M. Porsch-Ozucurumez, et al. 1999. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat. Genet.* **22:** 347–351.
3. Brooks-Wilson, A., M. Marcil, S. M. Clee, L-H. Zhang, K. Roomp, M. van Dam, C. Brewer, J. A. Collins, H. O. F. Molhuizen, D. Loubser, et al. 1999. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat. Genet.* **22:** 336–345.
4. Rust, S., M. Rosier, H. Funke, J. Real, Z. Amoura, J-C. Piette, J-F. Deleuze, H. B. Brewer, N. Duverger, P. Denefle, et al. 1999. Tangier disease is caused by mutations in the gene encoding ATP binding-cassette transporter 1. *Nat. Genet.* **22:** 352–355.
5. Vaisman, B. L., G. Lambert, M. Amar, C. Joyce, T. Ito, R. D. Shamburek, W. J. Cain, J. Fruchart-Najib, E. D. Neufeld, A. T. Remaley, et al. 2001. ABCA1 overexpression leads to hyperalphalipoproteinemia and increased biliary cholesterol excretion in transgenic mice. *J. Clin. Invest.* **108:** 303–309.
6. Singaraja, R. R., V. Bocher, E. R. James, S. M. Clee, L-H. Zhang, B. R. Leavitt, B. Tan, A. Brooks-Wilson, A. Kwok, N. Bissada, et al. 2001. Human ABCA1 BAC transgenic mice show increased high density lipoprotein cholesterol and apoAI-dependent efflux stimulated by an internal promoter containing liver X receptor response elements in intron 1. *J. Biol. Chem.* **276:** 33969–33979.
7. Yamauchi, Y., S. Abe-Dohmae, and S. Yokoyama. 2002. Differential regulation of apolipoprotein A-I/ATP binding cassette transporter A1-mediated cholesterol and phospholipid release. *Biochim. Biophys. Acta.* **1585:** 1–10.
8. Li, 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.
9. Li, 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.
10. 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.
11. Arakawa, R., S. Abe-Dohmae, M. Asai, J. Ito, and S. Yokoyama. 2000. Involvement of caveolin-1 in cholesterol enrichment of HDL during its assembly by apolipoprotein and THP-1 cells. *J. Lipid Res.* **41:** 1952–1962.
12. Sugimoto, K., M. Tsujita, C. A. Wu, K. Suzuki, and S. Yokoyama. 2004. An inhibitor of acylCoA:cholesterol acyltransferase increases expression of ATP-binding cassette transporter A1 and thereby enhances the apoA-I-mediated release of cholesterol from macrophages. *Biochim. Biophys. Acta.* **1636:** 69–76.
13. Ito, J., Y. Nagayasu, K. Kato, R. Sato, and S. Yokoyama. 2002. Apolipoprotein A-I induces translocation of cholesterol, phospholipid, and caveolin-1 to cytosol in rat astrocytes. *J. Biol. Chem.* **277:** 7929–7935.
14. Wang, N., D. L. Silver, P. Costet, and A. R. Tall. 2000. Specific binding of apoA-I, enhanced cholesterol efflux and altered plasma membrane morphology in cells expressing ABC1. *J. Biol. Chem.* **275:** 33053–33058.
15. Tanaka, A. R., S. Abe-Dohmae, T. Ohnishi, R. Aoki, G. Morinaga, K. Okuhira, Y. Ikeda, F. Kano, M. Matsuo, N. Kioka, et al. 2003. Effects of mutations of ABCA1 in the first extracellular domain on subcellular trafficking and ATP binding/hydrolysis. *J. Biol. Chem.* **278:** 8815–8819.
16. Abe-Dohmae, S., Y. Ikeda, M. Matsuo, M. Hayashi, K. Okuhira, K. Ueda, and S. Yokoyama. 2004. Human ABCA7 supports apolipoprotein-mediated release of cellular cholesterol and phospholipid to generate high density lipoprotein. *J. Biol. Chem.* **279:** 604–611.
17. Wang, N., D. Lan, M. Gerbod-Giannone, P. Linsel-Nitschke, A. W. Jehle, W. Chen, L. O. Martinez, and A. R. Tall. 2003. ATP-binding cassette transporter A7 (ABCA7) binds apolipoprotein A-I and mediates cellular phospholipid but not cholesterol efflux. *J. Biol. Chem.* **278:** 42906–42912.
18. Lawn, R. M., D. P. Wade, M. R. Garvin, X. 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.
19. 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.
20. 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.
21. Costet, P., Y. Luo, N. Wang, and A. R. Tall. 2000. Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J. Biol. Chem.* **275:** 28240–28245.
22. 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.

23. Arakawa, R., and S. Yokoyama. 2002. Helical apolipoproteins stabilize ATP-binding cassette transporter A1 by protecting it from thiol protease-mediated degradation. *J. Biol. Chem.* **277:** 22426–22429.
24. Costet, P., F. Lalanne, M. C. Gerbod-Giannone, J. R. Molina, X. Fu, E. G. Lund, L. J. Gudas, and A. R. Tall. 2003. Retinoic acid receptor-mediated induction of ABCA1 in macrophages. *Mol. Cell. Biol.* **23:** 7756–7766.
25. No, D., T. P. Yao, and R. M. Evans. 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. USA.* **93:** 3346–3351.
26. Tajima, S., S. Yokoyama, and A. Yamamoto. 1983. Effect of lipid particle size on association of apolipoproteins with lipid. *J. Biol. Chem.* **258:** 10073–10082.
27. Hara, H., and S. Yokoyama. 1991. Interaction of free apolipoprotein with macrophages: formation of high density lipoprotein-like lipoproteins and reduction of cellular cholesterol. *J. Biol. Chem.* **266:** 3080–3086.
28. Okazaki, M., S. Usui, and S. Hosaki. 2000. Analysis of plasma lipoproteins by gel permeation chromatography. *In* Handbook of Lipoprotein Testing. N. Rifai, G. R. Warnick, and M. H. Dominiczak, editors. American Association of Clinical Chemistry Press, Washington, DC. 647–669.
29. Usui, S., Y. Hara, S. Hosaki, and M. Okazaki. 2002. A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. *J. Lipid Res.* **43:** 805–814.
30. Hamon, Y., C. Broccardo, O. Chambenoit, M. F. Luciani, F. Toti, S. Chaslin, J. M. Freyssinet, P. F. Devaux, J. McNeish, D. Marguet, et al. 2000. ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. *Nat. Cell Biol.* **2:** 399–406.
31. Okazaki, M., S. Usui, M. Ishigami, N. Sakai, T. Nakamura, Y. Matsuzawa, and S. Yamashita. 2005. Identification of unique lipoprotein subclasses for visceral obesity by component analysis of cholesterol profile in high-performance liquid chromatography. *Arterioscler. Thromb. Vasc. Biol.* **25:** 578–584.
32. Linsel-Nitschke, P., A. W. Jehle, J. Shan, G. Cao, D. Bacic, D. Lan, N. Wang, and A. R. Tall. 2005. Potential role of ABCA7 in cellular lipid efflux to apoA-I. *J. Lipid Res.* **46:** 86–92.
33. Kim, W. S., M. L. Fitzgerald, K. Kang, K. Okuhira, S. A. Bell, J. J. Manning, S. L. Koehn, N. Lu, K. J. Moore, and M. W. Freeman. 2005. Abca7 null mice retain normal macrophage phosphatidylcholine and cholesterol efflux activity despite alterations in adipose mass and serum cholesterol levels. *J. Biol. Chem.* **280:** 3989–3995.
34. Suzuki, S., T. Nishimaki-Mogami, N. Tamehiro, K. Inoue, R. Arakawa, S. Abe-Dohmae, A. R. Tanaka, K. Ueda, and S. Yokoyama. 2004. Verapamil increases the apolipoprotein-mediated release of cellular cholesterol by induction of ABCA1 expression via liver X receptor-independent mechanism. *Arterioscler. Thromb. Vasc. Biol.* **24:** 519–525.
35. Chinetti, G., S. Lestavel, V. Bocher, A. T. Remaley, B. Neve, I. P. Torra, E. Teissier, A. Minnich, M. Jaye, N. Duverger, et al. 2001. PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat. Med.* **1:** 53–58.
36. Arakawa, R., N. Tamehiro, T. Nishimaki-Mogami, K. Ueda, and S. Yokoyama. 2005. Fenofibric acid, an active form of fenofibrate, increases apoAI-mediated HDL biogenesis by enhancing transcription of ABCA1 gene in an LXR-dependent manner. *Arterioscler. Thromb. Vasc. Biol.* **25:** 1193–1197.
37. Forte, T. M., R. Goth-Goldstein, R. W. Nordhausen, and M. R. McCall. 1993. Apolipoprotein A-I-cell membrane interaction: extracellular assembly of heterogeneous nascent HDL particles. *J. Lipid Res.* **34:** 317–324.
38. Liu, L., A. E. Bortnick, M. Nickel, P. Dhanasekaran, P. V. Subbaiah, S. Lund-Katz, G. H. Rothblat, and M. C. Phillips. 2003. Effects of apolipoprotein A-I on ATP-binding cassette transporter A1-mediated efflux of macrophage phospholipid and cholesterol: formation of nascent high density lipoprotein particles. *J. Biol. Chem.* **278:** 42976–42984.
39. Li, L., J. Chen, V. K. Mishra, J. A. Kurtz, D. Cao, A. E. Klon, S. C. Harvey, G. M. Anantharamaiah, and J. P. Segrest. 2004. Double belt structure of discoidal high density lipoproteins: molecular basis for size heterogeneity. *J. Mol. Biol.* **343:** 1293–1311.
40. Curtiss, L. K., D. J. Bonnet, and K. A. Rye. 2000. The conformation of apolipoprotein A-I in high-density lipoproteins is influenced by core lipid composition and particle size: a surface plasmon resonance study. *Biochemistry.* **39:** 5712–5721.
41. Vedhachalam, C., L. Liu, M. Nickel, P. Dhanasekaran, G. M. Anantharamaiah, S. Lund-Katz, G. H. Rothblat, and M. C. Phillips. 2004. Influence of apoA-I structure on the ABCA1-mediated efflux of cellular lipids. *J. Biol. Chem.* **279:** 49931–49939.
42. Vaughan, A. M., and J. F. Oram. 2003. ABCA1 redistributes membrane cholesterol independent of apolipoprotein interactions. *J. Lipid Res.* **44:** 1373–1380.
43. Denis, M., B. Haidar, M. Marcil, M. Bouvier, L. Krimbou, and J. Genest. 2004. Characterization of oligomeric human ATP binding cassette transporter A1. Potential implications for determining the structure of nascent high density lipoprotein particles. *J. Biol. Chem.* **279:** 41529–41536.

Fenofibric Acid, an Active Form of Fenofibrate, Increases Apolipoprotein A-I–Mediated High-Density Lipoprotein Biogenesis by Enhancing Transcription of ATP-Binding Cassette Transporter A1 Gene in a Liver X Receptor–Dependent Manner

Reijiro Arakawa, Norimasa Tamehiro, Tomoko Nishimaki-Mogami, Kazumitsu Ueda, Shinji Yokoyama

Objective—Fibrates are widely used drugs to reduce plasma triglyceride and increase high-density lipoprotein. Their active forms, fibric acids, are peroxisome proliferator-activated receptor-α activators, but no direct evidence has been demonstrated for their activation of ATP-binding cassette transporter A1 (ABCA1) in relation to clinically used fibrates. We investigated the reaction of fenofibric acid in this regard.

Methods and Results—Fenofibric acid was examined for the effect of increase of ABCA1 activity. It enhanced ABCA1 gene transcription and its protein level in macrophage cell line cells and fibroblasts and increased apolipoprotein A-I–mediated cellular lipid release, all in a dose-dependent manner. Enhancement of the gene transcription was examined by using a reporter assay system for liver X receptor responsive element (LXRE) and its inactive mutant. The results demonstrated that the effect of fenofibric acid is dependent on active LXRE.

Conclusions—Fenofibric acid increased transcription of ABCA1 gene in a liver X receptor–dependent manner. (*Arterioscler Thromb Vasc Biol.* **2005;25:1193-1197.**)

Key Words: fenofibrate ■ fibrates ■ PPARα ■ ABCA1 ■ HDL ■ cholesterol ■ atherosclerosis

High-density lipoprotein (HDL) is a negative risk factor in coronary atherogenesis,[1] and raising HDL is expected to protect us against atherosclerosis. Such an effect was demonstrated in experimental animals by specific gene expression[2] or inhibition of cholesteryl ester transfer protein (CETP).[3] Although no specific drug is available in clinical use for this purpose, a bile acid–sequestering resin and statins were shown to raise HDL by an unknown mechanism, besides lowering low-density lipoprotein, and subanalysis of these results indicated its independent effect of reducing the atherosclerosis risk.[4–6] Fibric acids, active forms of fibrate drugs and activators of peroxisome proliferator-activated receptor-α (PPARα),[7,8] are also known for an HDL-raising effect. This group of drugs has been widely used for a long time for the treatment of hyperlipoproteinemia, especially types IIb, III, and VI. Fibric acids enhance fatty acid catabolism and accordingly reduce plasma lipid level, predominantly triglyceride (TG). Increase of TG-rich lipoprotein results in increase of TG transfer to HDL in exchange with its cholesteryl ester by CETP, and therefore leads to production of small cholesterol-poor HDL as TG is hydrolyzed.[9,10] Consequently, reduction of TG-rich lipoprotein by fibrates leads to the increase of HDL cholesterol by reversing this mechanism.[11,12] Fibric acids were also shown to enhance transcription of the gene of apolipoprotein A-I (apoA-I) in the liver.[13–15] A PPARα activator, Wy14643, was shown to upregulate the gene of ATP-binding cassette transporter A1 (ABCA1)[16] that mediates and rate-limits biogenesis of HDL by the interaction of helical apolipoprotein and cells.[17–20] ABCA1 expression is enhanced by loading cholesterol to cells via the liver X receptor (LXR),[21,22] presumably because of the increase of oxysterol. The effect of Wy14643 was interpreted by the activation of the LXR pathway as it increased LXR.[16] However, there has been no direct demonstration of the ABCA1 upregulation by fibric acids derived from fibrate drugs clinically used. In mouse atherosclerosis models, PPARα agonists did not appear to enhance ABCA1 expression in atherosclerotic lesion despite their effect of the regression.[23,24] Here we report in vitro observation that fenofibric acid increases the expression of ABCA1 and apoA-I–mediated HDL production. The effect on ABCA1 expression was through the enhancement of the transcription of the ABCA1 gene being dependent on LXR.

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From the Department of Biochemistry, Cell Biology, and Metabolism (R.A., S.Y.), Nagoya City University Graduate School of Medical Sciences, Japan; Research and Development Institute (R.A.), Grelan Pharmaceutical Co., Tokyo, Japan; Division of Biosignaling (N.T., T.N.-M.), National Institute of Health Sciences, Tokyo, Japan; and Division of Applied Life Sciences (K.U.), Graduate School of Agriculture, Kyoto University, Japan.

Correspondence to Shinji Yokoyama, Department of Biochemistry, Cell Biology, and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan. E-mail syokoyam@med.nagoya-cu.ac.jp

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Materials and Methods

Cell Culture

RAW264 cells were maintained in Dulbecco modified Eagle medium (DMEM)/F-12 (1:1) medium (IWAKI Glass) containing 2% TCM serum replacement (purchased from KN) at 37°C in 5% CO_2.[25] Cells in 6-well plates at the concentration of 1.5×10^6 cells per well were incubated 24 hours before the experiments.[25] THP-1 cells (4.0×10^6 cells per well) were differentiated with 3.2×10^{-7} M phorbol 12-myristate 13-acetate (PMA; Wako) in 10% FBS (PAA Laboratories)-RPMI 1640 medium (IWAKI Glass) for 72 hours.[26] BALB/3T3 clone A31[27] (obtained from RIKEN Cell Bank) was incubated in Eagle's minimum essential medium (MEM) with 10% FCS. All cell lines were incubated at 37°C in a humidified atmosphere of 5% CO_2. PPARα activators, fenofibric acid, or Wy14643 (Calbiochem-Novabiochem) were dissolved in dimethyl sulfoxide and added to the culture medium containing 0.2% BSA (Sigma).

Cellular Lipid Release

RAW264 cells were washed with PBS and cultured an additional 48 hours in the presence of fenofibric acid or Wy14643 in DMEM/F-12 (1:1) medium containing 2% TCM and 0.2% BSA. During the last 24 hours of the drug treatment, 300 μmol/L of dibutyryl cAMP (dbcAMP; Wako) and apoA-I (10 μg/mL) were added to the medium.[25] THP-1 cells and BALB/3T3 cells were also treated with the PPARα activators and apoA-I in 0.2% BSA-RPMI 1640 medium and 0.1% BSA-MEM, respectively. Cholesterol and choline-phospholipid released into the medium by apoA-I were determined enzymatically.[25] Adherent cells were dissolved in 0.1 N NaOH for protein determination by bicinchoninic acid protein assay system (Pierce).

Reporter Gene Assay

The constructs of luciferase reporter genes were prepared as described previously.[28] The 5′-flanking region of mouse ABCA1 gene (−1238/+57) was inserted into pGL3 vector (Promega) to generate ABCA1 promotor-luciferase reporter construct (pABCA1-Luc). The reporter plasmid with mutated and inactivated LXR-responsive element (LXRE) (mutant LXRE) was generated by using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mutations introduced were identical to those reported previously.[21] Cells cultured in 24-well plates (3.0×10^3 cells per well) were washed once with PBS or pABCA1-Luc vector or pABCA1-mutant LXRE-Luc vector were cotransfected with phRL-tk vector (Promega) by Superfect transfection reagent (Qiagen). Three hours after the transfection, cells were washed with PBS and cultured in the presence of fenofibric acid or Wy14643 for 24 hours. Cellular luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega). Results were standardized by the Renilla luciferase activity derived from phRL-tk vector.

Immunoblotting of ABCA1

Cells incubated with fenofibrate or Wy14643 for 48 hours were harvested in cold PBS and pelleted by centrifugation. The cell pellet was suspended in 5 mmol/L Tris-HCl, pH 8.5, containing 1% protease inhibitor cocktails (Sigma) and placed on ice for 30 minutes. The cell suspension was centrifuged at 650*g* for 5 minutes, and the supernatant was centrifuged at 105 000*g* for 30 minutes to prepare the membrane fraction as a pellet. Immunoblotting of ABCA1 was performed according to the previous method.[26]

Results

Expression of ABCA1

The effects of fenofibric acid and Wy14643 on expression of ABCA1 are shown in Figures 1 and 2. The message of ABCA1 increased by fenofibric acid and Wy14643 in all types of cells examined: RAW264 cells treated with dbcAMP, PMA-differentiated THP-1 cells, and BALB/3T3 cells. ABCA1 protein also increased by the PPARα agonists being demonstrated by its immunoblotting analysis in all these cells (Figures 1 and 2).

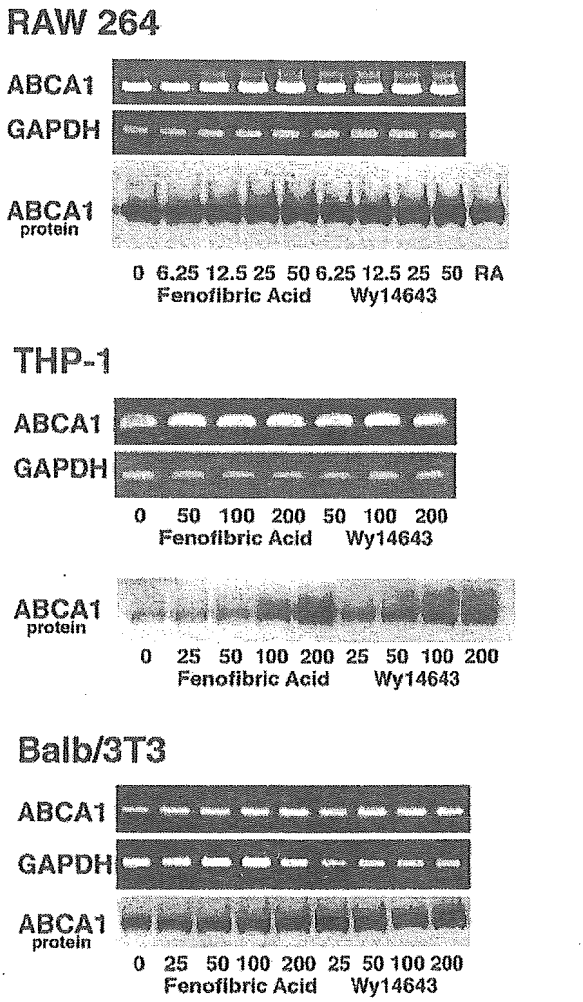

Figure 1. Effects of PPARα agonists fenofibric acid and Wy14643 on expression of ABCA1 in RAW264 cells pretreated with dbcAMP and THP-1 cells differentiated with PMA and BALB/3T3 fibroblasts. Messages of ABCA1 and GAPDH were detected by RT-PCR, and protein level of ABCA1 was determined by immunoblotting, as described in the text in each type of cell in the presence of the agonists (μmol/L) and 9-*cis*-retinoic acid (RA).

ApoA-I–Mediated Cellular Lipid Release

ApoA-I induced release of cellular cholesterol and phospholipids into the medium from the cells examined. PPARα agonists fenofibric acid and Wy14643 increased the apoA-I–mediated release of cholesterol and phospholipids in a dose-dependent manner (Figure 3). The increment of lipids released by the drugs was more prominent in cholesterol than phospholipid in RAW264 cells pretreated by dbcAMP. The maximum effect (102% increase in cholesterol release) was observed when the cells were treated with 25 μmol/L of fenofibric acid.

Reporter Gene Assay

Transcription of the ABCA1 gene was examined by using the reporter genes (pABCA1-Luc) in the dbcAMP-treated RAW264 cells (Figure 4). Fenofibric acid and Wy14642

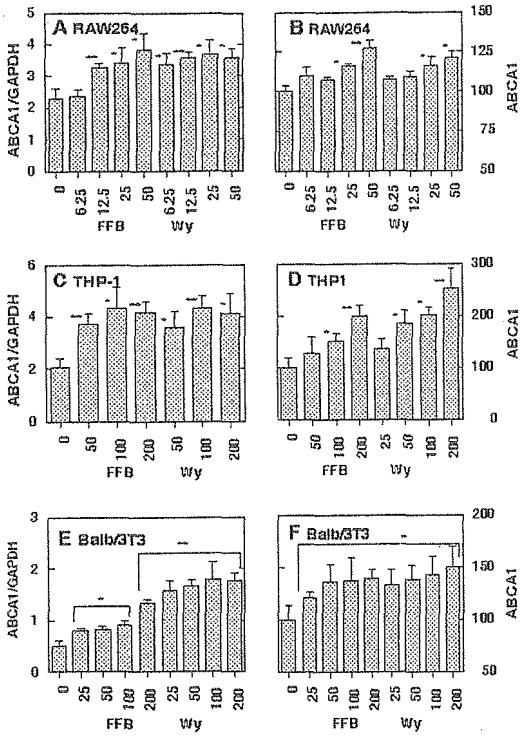

Figure 2. Effects of PPARα agonists fenofibric acid (FFB; μmol/L) and Wy14643 (Wy; μmol/L) on expression of ABCA1. The results of RT-PCR and Western blotting from the same experiments shown in Figure 1 were semiquantified by digital scanning in an Epson GT9500. Message of ABCA1 was standardized for that of GAPDH. Data points represent mean±SE of 3 independent experiments. Significance of the increase from the controls was examined by Student's *t* test and indicated as $^{*}P<0.05$ and $^{**}P<0.01$.

enhanced transcription of the ABCA1 reporter gene in a dose-dependent manner (Figure 4A and 4B). These effects were cancelled by substitute transfection of the mutant LXRE-containing reporter vector (pABCA1-mutant LXRE-

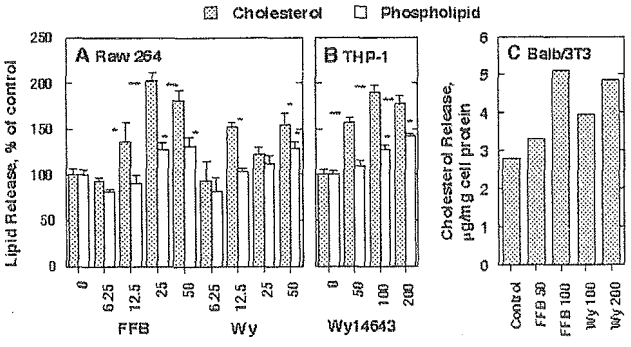

Figure 3. Lipid release by apoA-I from the cells examined in the presence of PPARα agonists fenofibric acid (FFB; μmol/L) and Wy14643 (Wy; μmol/L). Releases of cholesterol and phospholipid are expressed as percentage of the control for RAW264 cells (cholesterol 2.39 μg/mg cell protein and phospholipid 5.89 μg/mg cell protein), and for THP-1 cells (cholesterol 2.45 μg/mg cell protein and phospholipid 4.26 μg/mg cell protein). Data points in A and B represent mean±SE of triplicate measurement, and those in C represent the average of duplicate measurement. Significance of the increase from the controls was examined using Student's *t* test and indicated as $^{*}P<0.05$ and $^{**}P<0.01$.

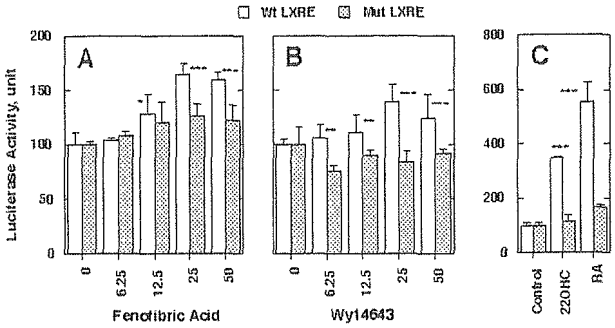

Figure 4. Luciferase reporter gene assay of ABCA1. The reporter genes for the ABCA1 promoter were constructed as described in the text. Wt-LXRE and Mut-LXRE indicate the genes without and with introduction of mutation in LXRE to inactivate the responsive element. The effects of fenofibric acid (FFB; μmol/L) and Wy14643 (Wy; μmol/L) were examined, as well as those of 9-*cis*-retionic acid (RA) and 22-oxysterol (22OHC). Data points represent mean±SE of triplicate measurement. Significance of the increase was examined using Student's *t* test and indicated as $^{*}P<0.05$ from the blanks (no compound), $^{**}P<0.05$ from the controls (mutant LXRE), and $^{***}P<0.05$ *from both.*

Luc) to inactivate LXRE (Figure 4A and 4B), whereas 9-*cis*-retionic acid, a ligand for retinoid X receptor (RXR), and 22-oxysterol, a ligand for LXR, failed to increase the transcription of the mutant ABCA1 gene (Figure 4C).

Discussion

PPARs belong to the nuclear receptor superfamily group and act as ligand-activated transcription factors regulating the expression of certain target genes.[29] The PPAR family contains 3 different subtypes, designated PPARα, PPARβ/δ, and PPARγ. PPAR subtypes display distinct expression patterns, different ligand specificities, and distinct biological functions.[30–32] PPARs are activated by fatty acids and its metabolites and accordingly exert various effects in lipid homeostasis.[33] Several subtype-specific synthetic compounds have been developed for clinical use, including fibric acids (PPARα agonist) and glitazones (PPARγ agonist).[34] Fibrates are widely used drugs for hyperlipidemic patients because they significantly improve plasma lipid profiles by reducing TG-rich lipoprotein and raising HDL.[35,36] The primary effects of fibric acids, active forms of fibrates, on plasma lipids have been attributed to their PPARα-mediated expression of the genes of various enzymes that regulate lipid metabolism.[7,8] For HDL metabolism, the effects are partly explained by reduction of plasma TG itself and CETP reaction[9,10] and by increased expression of the apoA-I gene.[13–15] In addition, Wy14643, a nonclinical PPARα activator, was shown to enhance ABCA1 gene expression.[16] Because LXR was also activated in the condition used in that work,[16] and ABCA1 is known to be regulated by the LXR/RXR pathway, it was hypothesized that Wy14643 increases the transcription of ABCA1 gene via the LXR pathway.

We demonstrated the increase of ABCA1 by fenofibric acid, an active form of clinically used fibrate drug fenofibrate, in macrophage cell line cells and in mouse fibroblasts. These effects were also reproduced by a positive control Wy14643. To examine the mechanism, the reporter gene

assay was used via a promoter of the ABCA1 gene by introducing a mutation in LXR response element. Inactivation of this element was verified by abolishment of its response to 9-*cis* retinoic acid and 22-oxysterol, and fenofibrate failed to enhance transcription of the mutant reporter gene. Therefore, PPARα in fact activates the ABCA1 gene by the LXR-dependent pathway. The results were inconsistent with the finding that PPARs form a heterodimeric complex with the RXR (not LXR) and bind to specific PPAR-response elements in the promoter region of target gene.[37,38] However, a direct ligand of RXR, 9-*cis* retinoic acid, failed to activate the mutant gene, consistent with the established finding that dimerization of RXR with LXR is essential for enhancing ABCA1 gene transcription.[21]

Fenofibrate and Wy14643 reportedly have different affinity and distinct specificity to murine and human PPARs. However, both compounds showed equivalent capability in transactivation of the ABCA1 gene. Wy14643 activates not only PPARα but also PPARγ and PPARδ in cell-based transactivation assays[39] at the concentration >30 μmol/L. Activation of PPARδ was suggested to affect the ABCA1-mediated HDL biogenesis on the basis that an agonist of PPARδ induced HDL synthesis in culture cells and in monkeys.[40] Therefore, the effects of Wy14643 may include combined activation of various PPARs. In contrast, fenofibric acid is highly specific for activation of PPARα, at least up to 100 μmol/L.[39] Because C_{max} of fenofibrae is 30 μmol/L when it is orally administrated to human, it is most likely that the effect of this drug on the HDL biogenesis is based on the enhancement of ABCA1 expression by the mechanism shown in this article.

Fenofibrate has been shown to retard progression of coronary atherosclerosis,[41] consistent with the findings of reducing a risk of coronary heart disease by other fibrate drugs.[42,43] The clinical effects of these drugs are attributed to improvement of plasma lipoprotein profile by reducing TG and raising HDL. Although decrease of TG and increase of HDL are linked in human by the action of CETP,[10] the increase of ABCA1 activity may more directly contribute to raising HDL and prevention of lipid accumulation in vascular cells.

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References

1. Gordon DJ, Rifkind BM. High-density lipoprotein—the clinical implications of recent studies. *New Eng J Med.* 1989;321:1311–1316.
2. Rubin EM, Krauss RM, Spangler EA, Verstuyft JG, Clift SM. Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI. *Nature.* 1991;353:265–267.
3. Okamoto H, Yonemori F, Wakitani K, Minowa T, Maeda K, Shinkai H. A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. *Nature.* 2000;406:203–207.
4. Gordon DJ, Knoke J, Probstfield JL, Superko R, Tyroler HA. High-density lipoprotein cholesterol and coronary heart disease in hypercholesterolemic men: the Lipid Research Clinics Coronary Primary Prevention Trial. *Circulation.* 1986;74:1217–1225.
5. Vrecer M, Turk S, Drinovec J, Mrhar A. Use of statins in primary and secondary prevention of coronary heart disease and ischemic stroke. Meta-analysis of randomized trials. *Int J Clin Pharmacol Ther.* 2003;41:567–577.
6. Athyros VG, Mikhailidis DP, Papageorgiou AA, Symeonidis AN, Mercouris BR, Pehlivanidis A, Bouloukos VI, Elisaf M, Group GC; GREASE Collaborative Group. Effect of atorvastatin on high-density lipoprotein cholesterol and its relationship with coronary events: a subgroup analysis of the GREek Atorvastatin and Coronary-heart-disease Evaluation (GREACE) Study. *Curr Med Res Opin.* 2004;20:627–637.
7. Issemann I, Green S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature.* 1990;347:645–650.
8. Yadetie F, Laegreid A, Bakke I, Kusnierczyk W, Komorowski J, Waldum HL, Sandvik AK. Liver gene expression in rats in response to the peroxisome proliferator-activated receptor-alpha agonist ciprofibrate. *Physiol Genomics.* 2003;15:9–19.
9. Ko KW, Ohnishi T, Yokoyama S. Triglyceride transfer is required for net cholesteryl ester transfer between lipoproteins in plasma by lipid transfer protein. Evidence for a hetero-exchange transfer mechanism demonstrated by using novel monoclonal antibodies. *J Biol Chem.* 1994;269:28206–28213.
10. Foger B, Ritsch A, Doblinger A, Wessels H, Patsch JR. Relationship of plasma cholesteryl ester transfer protein to HDL cholesterol. Studies in normotriglyceridemia and moderate hypertriglyceridemia. *Arterioscler Thromb Vasc Biol.* 1996;16:1430–1436.
11. Despres JP. Increasing high-density lipoprotein cholesterol: an update on fenofibrate. *Am J Cardiol.* 2001;88:30N–36N.
12. Pauciullo P, Marotta G, Rubba P, Cortese C, Caruso MG, Gnasso A, Fischetti A, Motti C, Mancini M. Serum lipoproteins, apolipoproteins and very low density lipoprotein subfractions during 6-month fibrate treatment in primary hypertriglyceridemia. *J Intern Med.* 1990;228:425–430.
13. Berthou L, Duverger N, Emmanuel F, Langouet S, Auwerx J, Guillouzo A, Fruchart JC, Rubin E, Denefle P, Staels B, Branellec D. Opposite regulation of human versus mouse apolipoprotein A-I by fibrates in human apolipoprotein A-I transgenic mice. *J Clin Invest.* 1996;97:2408–2416.
14. Krause BR, Newton RS. Gemfibrozil increases both apo A-I and apo E concentrations. Comparison to other lipid regulators in cholesterol-fed rats. *Atherosclerosis.* 1986;59:95–98.
15. Staels B, Dallongeville J, Auwerx J, Schoonjans K, Leitersdorf E, Fruchart JC. Mechanism of action of fibrates on lipid and lipoprotein metabolism. *Circulation.* 1998;98:2088–2093.
16. Chinetti G, Lestavel S, Bocher V, Remaley AT, Neve B, Torra IP, Teissier E, Minnich A, Jaye M, Duverger N, Brewer HB, Fruchart JC, Clavey V, Staels B. PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat Med.* 2001;7:53–58.
17. Hara H, Yokoyama S. Interaction of free apolipoproteins with macrophages: Formation of high-density lipoprotein-like lipoproteins and reduction of cellular cholesterol. *J Biol Chem.* 1991;266:3080–3086.
18. Bodzioch M, Orso E, Klucken J, Langmann T, Bottcher A, Diederich W, Drobnik W, Barlage S, Buchler C, Porsch-Ozcurumez M, Kaminski WE, Hahmann HW, Oette K, Rothe G, Aslanidis C, Lackner KJ, Schmitz G. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat Genet.* 1999;22:347–351.
19. Brooks-Wilson A, Marcil M, Clee SM, Zhang L-H, Roomp K, van Dam M, Yu L, Brewer C, Collins JA, Molhuizen HOF, Loubser O, Ouelette BFF, Fichter K, Ashbourne-Excoffon KJD, Sensen CW, Scherer S, Mott S, Denis M, Martindale D, Frohlich J, Morgan K, Koop B, Pimstone S, Kastelein JJP, Genest J Jr, Hayden MR. Mutations in ABC 1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat Genet.* 1999;22:336–345.
20. Rust S, Rosier M, Funke H, Real J, Amoura Z, Piette J-C, Deleuze J-F, Brewer HB, Duverger N, Denefle P, Assmann G. Tangier disease is caused by mutations in the gene encoding ATP binding-cassette transporter 1. *Nat Genet.* 1999;22:352–355.
21. Costet P, Luo Y, Wang N, Tall AR. Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J Biol Chem.* 2000;275:28240–28245.
22. Schwartz K, Lawn RM, Wade DP. ABC1 gene expression and ApoA-I–mediated cholesterol efflux are regulated by LXR. *Biochem Biophys Res Commun.* 2000;274:794–802.
23. Duez H, Chao Y-S, Hernandez M, Torpier G, Poulain P, Mundt S, Mallat Z, Teissier E, Burton CA, Tedgui A, Fruchart J-C, Fievet C, Wright SD, Staels B. Reduction of atherosclerosis by the peroxisome proliferator-ac-

tivated receptor agonist fenofibrate in mice. *J Biol Chem.* 2002;277: 48051–48057.
24. Li AC, Binder CJ, Gutierrez A, Brown KK, Plotkin CR, Pattison JW, Valledor AF, Davis RA, Willson TM, Witztum JL, Palinski W, Glass CK. Differential inhibition of macrophage foam-cell formation and atherosclerosis in mice by PPARα, β/δ, and γ. *J Clin Invest.* 2004;114:1564–1576.
25. Abe-Dohmae S, Suzuki S, Wada Y, Aburatani H, Vance DE, Yokoyama S. Characterization of apolipoprotein-mediated HDL generation induced by cAMP in a murine macrophage cell line. *Biochemistry.* 2000;39: 11092–11099.
26. Arakawa R, Abe-Dohmae S, Asai M, Ito J, Yokoyama S. Involvement of caveolin-1 in cholesterol-enrichment of HDL during its assembly by apolipoprotein and THP-1 cells. *J Lipid Res.* 2000;41:1952–1962.
27. Kakunaga T. A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from BALB-3T3. *Int J Cancer.* 1973;12:463–473.
28. Suzuki S, Nishimaki-Mogami T, Tamehiro N, Inoue K, Arakawa R, Abe-Dohmae S, Tanaka AR, Ueda K, Yokoyama S. Verapamil increases the apolipoprotein-mediated release of cellular cholesterol by induction of ABCA1 expression via liver X receptor-independent mechanism. *Arterioscler Thromb Vasc Biol.* 2004;24:519–525.
29. Torra IP, Chinetti G, Duval C, Fruchart J-C, Staels B. Peroxisome proliferator-activated receptors: from transcriptional control to clinical practice. *Curr Opin Lipidol.* 2001;12:245–254.
30. Kliewer SA, Umesono K, Noonan DJ, Heyman RA, Evans RM. Convergence of 9-*cis* retinoic acid and peroxisome proliferator signaling pathways through heterodimer formation of their receptors. *Nature.* 1992; 358:771–774.
31. Kliewer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, Umesono K, Evans RM. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc Natl Acad Sci U S A.* 1994;91:7355–7359.
32. Braissant O, Foufelle F, Scotto C, Dauca M, Wahli W. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-α, -β, and -γ in the adult rat. *Endocrinology.* 1996;137:354–366.
33. Keller H, Dreyer C, Medin J, Mahfoudi A, Ozato K, Wahli W. Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc Natl Acad Sci U S A.* 1993;90:2160–2164.
34. Chakrabarti R, Vikramadithyan RK, Misra P, Hiriyan J, Raichur S, Damarla RK, Gershome C, Suresh J, Rajagopalan R. Ragaglitazar: a novel PPAR alpha PPAR gamma agonist with potent lipid-lowering and insulin-sensitizing efficacy in animal models. *Br J Pharmacol.* 2003;140: 527–537.
35. Hunninghake DB, Peters JR. Effect of fibric acid derivatives on blood lipid and lipoprotein levels. *Am J Med.* 1987;83:44–49.
36. Malmendier CL, Delcroix C. Effects of fenofibrate on high and low-density lipoprotein metabolism in heterozygous familial hypercholesterolemia. *Atherosclerosis.* 1985;55:161–169.
37. Miyata KS, McCaw SE, Marcus SL, Rachubinski RA, Capone JP. The peroxisome proliferator-activated receptor interacts with the retinoid X receptor in vivo. *Gene.* 1994;148:327–330.
38. Bardot O, Aldridge TC, Latruffe N, Green S. PPAR-RXR heterodimer activates a peroxisome proliferator response element upstream of the bifunctional enzyme gene. *Biochem Biophys Res Commun.* 1993;192: 37–45.
39. Fruchart JC, Staels B, Duriez P. The role of fibric acids in atherosclerosis. *Curr Atheroscler Rep.* 2001;3:83–92.
40. Oliver WR Jr, Shenk JL, Snaith MR, Russell CS, Plunket KD, Bodkin NL, Lewis MC, Winegar DA, Sznaidman ML, Lambert MH, Xu HE, Sternbach DD, Kliewer SA, Hansen BC, Willson TM. A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. *Proc Natl Acad Sci U S A.* 2001;98:5306–5311.
41. Effect of fenofibrate on progression of coronary-artery disease in type 2 diabetes: the Diabetes Atherosclerosis Intervention Study, a randomised study. *Lancet.* 2001;357:905–910.
42. Rubins HB, Robins SJ, Collins D, Fye CL, Anderson JW, Elam MB, Faas FH, Linares E, Schaefer EJ, Schectman G, Wilt TJ, Wittes J. Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial Study Group. *N Engl J Med.* 1999;341:410–418.
43. Secondary prevention by raising HDL cholesterol and reducing triglycerides in patients with coronary artery disease: the Bezafibrate Infarction Prevention (BIP) study. *Circulation.* 2000;102:21–27.

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REVIEW

Kei Takahashi · Yasuhisa Kimura · Koh Nagata
Akitsugu Yamamoto · Michinori Matsuo
Kazumitsu Ueda

ABC proteins: key molecules for lipid homeostasis

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Abstract Forty-nine ABC protein genes exist on human chromosomes. Eukaryotic ABC proteins were originally recognized as drug efflux pumps involved in the multidrug resistance of cancer cells. However, it is now realized that one of their major physiological roles is cellular lipid transport and homeostasis, and their dysfunction is often associated with human diseases. ABCA1 and ABCA7 mediate the apolipoprotein-dependent formation of a high-density lipoprotein–cholesterol complex. ABCA3 is indispensable for pulmonary surfactant secretion. ABCG5 and ABCG8 are involved in the secretion of plant sterols and cholesterol into bile. However, the primary substrates and mechanism of action of these ABC proteins have not been precisely defined. In this review article, we first describe the general structure and functions of eukaryotic ABC proteins. The current model of ABCA1 functionality is then explained based on studies on a topological model, subcellular localization, apoA-I dependence of HDL formation, functional defects of Tangier disease mutants, and ATP hydrolysis of purified ABCA1. ABCA1 is supposed to function as a transporter of lipids as well as a receptor for apoA-I. ABCA3 is likely involved in accumulating phospholipids and cholesterol in lamellar bodies and in generating multivesicular structures.

Key words Cholesterol · Transporter · ABCA1 · ABCA3 · ABCA7

Introduction

Eukaryotic ATP-binding cassette (ABC) proteins are generally recognized as drug efflux pumps that protect the body from various toxic substances. It is partly for historical reasons that *ABCB1* (*MDR1*), encoding P-glycoprotein, was the first of the ABC proteins to be identified in eukaryotes,[1–4] and another ABC protein gene *ABCC1* (*MRP1*) was isolated from multidrug resistant cancer cells[5] shortly after ABCB1. Both ABC proteins function as drug efflux pumps that protect the body from toxic substances. MDR1 extrudes a very wide array of structurally dissimilar compounds, all lipophilic and ranging in mass from approximately 300 to 2000 Da, including cytotoxic drugs that act on different intracellular targets. On the other hand, MRP1 transports various organic anionic conjugates, including glutathione, glucuronide, and sulfate conjugates. The discovery of MDR1 and MRP1 had a strong impact on the field of cancer chemotherapy and pharmacodynamics. This may be another reason for the strong impression that ABC proteins function as drug transporters. However, recent findings have suggested that the physiological role as self-defense machinery against xenobiotics is only one aspect of the importance of ABC proteins. The physiological importance of ABC proteins in lipid homeostasis has become obvious recently.

The importance of ABC proteins in lipid homeostasis was first suggested in 1993 with the discovery that ABCB4 (MDR2, also called MDR3) is a phosphatidylcholine translocator, indispensable for bile formation.[6] Two closely linked genes (*MDR1* and *MDR2*, also called *MDR3*) exist on chromosome 7 in humans, and three genes in mice (*mdr1*, *mdr2*, and *mdr3*, or *mdr1b*, *mdr2*, and *mdr1a*, respectively). Human *MDR1* and mouse *mdr1* and *mdr3* encode multidrug transporters whereas human MDR2 (MDR3) and mouse mdr2 are involved in phosphatidylcholine secretion into bile. The *ABCB4* gene is highly homologous to *ABCB1*, and exists next to *ABCB1* on chromosome 7q. The importance of ABC proteins in lipid homeostasis was firmly established by the discovery in 1999 that mutations in ABCA1 cause Tangier disease, a rare inherited disorder characterized by an absence of circulating high-density lipoprotein (HDL).[7,8]

Among 49 human ABC proteins, there still exist many whose physiological functions or endogenous substrates

K. Takahashi · Y. Kimura · K. Nagata · A. Yamamoto · M. Matsuo · K. Ueda (✉)
Laboratory of Cellular Biochemistry, Division of Applied Life Sciences, Faculty of Agriculture, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan
Tel. +81-75-753-6124; Fax +81-75-753-6104
e-mail: uedak@kais.kyoto-u.ac.jp

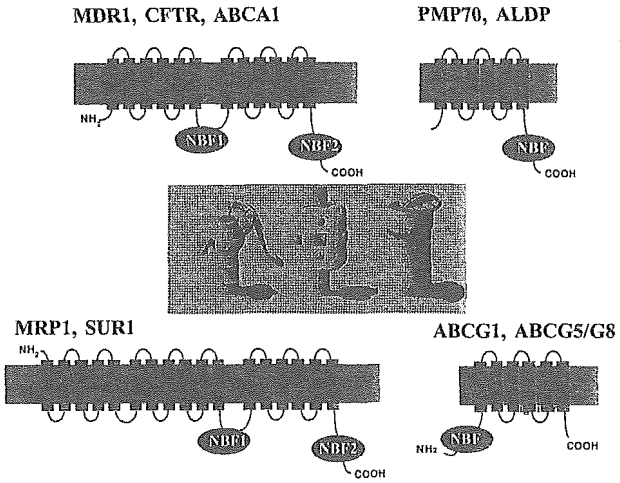

Fig. 1. Domain organization of eukaryotic ATP-binding cassette (ABC) proteins and drawings representing the three types of ABC proteins

are unclear. Also, awareness of the physiological importance of ABC proteins in lipid homeostasis is increasing. In this review article, we first describe the general structure and functions of ABC proteins and, in the second half, functions of ABCA subfamily proteins involved in lipid homeostasis.

Three major groups of eukaryotic ABC proteins

In eukaryotes, ABC proteins can be classified into three major groups based on function: transporters, regulators, and a channel (Fig. 1). Cystic fibrosis transmembrane conductance regulator (CFTR) is the only ABC protein clearly proven to function as a channel.[9–11] CFTR is a voltage-independent Cl^- channel found in the epithelial cells of many tissues and plays a major role in regulating Cl^- flux. Mutations in *CFTR* cause cystic fibrosis, one of the most common serious diseases, which affects 1 in 2000–2500 people in northern Europe and the United States. In addition to its Cl^- channel activity, CFTR is also suggested to act as a regulator of both an outwardly rectifying Cl^- channel (ORCC) and an epithelial Na^+ channel. Numerous abnormalities of cystic fibrosis are believed to be related to this multifunctionality of CFTR.[12]

The sulfonylurea receptor (SUR1) was identified as a target protein for sulfonylureas, such as glibenclamide, which is most commonly used in the treatment of noninsulin-dependent diabetes mellitus.[13] SUR1 is a subunit of the pancreatic β-cell K_{ATP} channel. The β-cell K_{ATP} channel is a hetero-octamer composed of pore-forming Kir6.2 subunits and SUR1 that coassemble with a 4:4 stoichiometry (5–8) and plays a key role in the regulation of glucose-induced insulin secretion. SUR1 is suggested to be not a channel or transporter itself but a switch that regulates the opening and closing of Kir6.2 channel subunits by monitoring the intracellular metabolic state, especially the ADP concentration.[14,15]

Most of the other eukaryotic ABC proteins seem to be transporters, although there are still many members with unknown functions. All the eukaryotic transporter-type ABC proteins studied to date transport substrates outwardly from cells. One exception may be a plant MDR-type ABC protein CjMDR1, which is involved in alkaloid transport in *Coptis japonica*, a perennial medicinal plant.[16] It is proposed that CjMDR1 is involved in the translocation of berberine from the root to the rhizome and functions as an influx pump for berberine.

Structure of ABC proteins

Domain structure

Most eukaryotic ABC proteins consist of four distinct domains (see Fig. 1). Two of these are highly hydrophobic, integral transmembrane domains (TMDs), each of which spans the membrane six times via α-helices. The other two are hydrophilic nucleotide-binding folds (NBFs), which share homology with potential nucleotide-binding sites of peripheral membrane components of bacterial active transporter systems. The individual domains are frequently expressed as separate polypeptides in prokaryotic ABC proteins,[17] whereas many eukaryotic ABC proteins have all four domains fused into a single polypeptide, as illustrated in Fig. 1.

Conserved sequence motifs

All the ABC proteins contain within each NBF at least three highly conserved sequence motifs, Walker A, Walker B, and the ABC signature sequence, known also as the C motif or a linker sequence. Walker A and B motifs are wildly conserved also among many nucleotide-binding proteins, such as ras P21 (Ras), RecA, adenylate kinase, myosin, and F_1-ATPase. The Walker A motif (G-X-X-G-X-G-K-S/T-S/T) is also known as a phosphate-binding loop (P-loop) or a glycine-rich loop. Residues within this motif interact with the phosphate groups and the magnesium ion of the bound Mg^{2+}–nucleotide complex. The Walker B motif is h-h-h-h-D, where h is a hydrophobic residue. In ATP- or GTP-binding proteins, this sequence constitutes a buried β-strand within the core of the NBF.[18] The highly conserved aspartate residue is involved in the coordination of the catalytic Mg^{2+} ion. An amino acid substitution of the conserved lysine residue in the Walker A motif or the conserved aspartate residue in the Walker B motif in either NBF results in a loss of the ATP hydrolysis activity of MDR1/P-glycoprotein.[19–21] The glutamate residue next to the aspartate residue of the Walker B motif is conserved among the ABC proteins except TAP1, NBF1s of MRPs, CFTR, or SURs. An amino acid substitution of this glutamate residue in the h-h-h-h-D-E sequence in either of the NBFs also abrogates steady-state ATP hydrolysis and

drug transport activities of MDR1 but does not impair vanadate-dependent nucleotide trapping.[22] An aspartate to glutamate substitution in NBF1 of MRP1 increases affinity for ADP and enhances ATP hydrolysis, whereas a glutamate to aspartate substitution in NBF2 of MRP1 decreases affinity for ATP and decreases ATP hydrolysis.[23] These results suggest that the acidic residue adjacent to the Walker B motif is involved in nucleotide binding, hydrolysis, and nucleotide release from NBF.

The C motif (L-S-G-G-Q-Q/R/K-Q-R) exists within each NBF of all the ABC proteins, but not of other nucleotide-binding protein families, such as GTP-binding proteins and the AAA superfamily. Therefore, it is also called the ABC signature motif. This motif is located immediately N-terminal to the Walker B motif. The C motif is suggested to be involved in the transduction of the energy of ATP hydrolysis to the conformational changes in the membrane integral domains required for translocation of the substrate.[17] According to the crystal structure of the *Escherichia coli* BtuCD protein, an ABC transporter mediating vitamin B_{12} uptake,[24] the two ATP-binding subunits (BtuD) are in close contact with each other in the crystal structure as predicted,[25] and the ATP molecules are proposed to be sandwiched between the Walker A sequence and the ABC signature motif of opposing BtuD subunits. This model is also supported by the structure of other prokaryotic ABC proteins such as MJ0796 and MalK.[26,27]

Other than the three conserved motifs mentioned above, glutamine located between the Walker A motif and the signature motif is highly conserved among the ABC proteins, and the loop containing this glutamine is called the Q-loop. A comparable amino acid substitution, Q1291R, in CFTR was observed in patients with cystic fibrosis, and the Q1291R CFTR shows no chloride channel function although it reaches the plasma membrane as a fully glycosylated mature protein.[28] A missense mutation of the corresponding glutamine in the second NBF of multidrug resistance protein 2 (MRP2, ABCC2) was detected in a patient with Dubin–Johnson syndrome (DJS), a hereditary disease characterized by hyperbilirubinemia. This mutation caused a lack of substrate-induced vanadate trapping, which may suggest that the glutamine is involved in the conformational change after ATP hydrolysis.[29]

Three-dimensional structure

An approximately 10-angstrom resolution structure of ABCB1 (MDR1/P-glycoprotein) was determined by electron cryomicroscopy of two-dimensional crystals.[30] The three-dimensional structure in the presence and absence of nucleotide was reported at a resolution of approximately 20 angstrom.[31] The TMDs form a chamber within the membrane that appears to be open to the extracellular milieu and may also be accessible from the lipid phase at the interfaces between the two TMDs. This structure may be consistent with a predicted model for the function of MDR1 and the general architecture of ABC proteins. MDR1 is predicted to act as a "vacuum cleaner" or a "flippase," with drug substrates gaining access to their binding site(s) from the inner leaflet of the lipid bilayer.[32] A gap present in the protein ring could allow substrates to access the central pore from the lipid phase. The substrates transported by different ABC proteins can vary widely, from small ions to large polypeptides and polysaccharides. A large pore could readily be adapted to accommodate different-sized substrates. The projection structures of MDR1 trapped at different steps of the transport cycle were also determined.[30] ATP binding, not hydrolysis, was proposed to drive the major conformational change associated with solute translocation. The crystal structure of the *E. coli* BtuCD protein, an ABC transporter mediating vitamin B_{12} uptake, was reported at a resolution of 3.2 angstroms.[24] The two ATP-binding subunits (BtuD) are in close contact with each other, as are the two membrane-spanning subunits (BtuC). However, because the BtuC subunits provide 20 transmembrane helices, we cannot predict the structure of eukaryotic ABC proteins directly from this conformation. Crystal structures at a higher resolution are prerequisite to understand the mechanism of substrate recognition and transport.

ABC proteins and lipid homeostasis

It is now realized that one of the major physiological roles of ABC transporters is cellular lipid transport and homeostasis, and their dysfunction is often associated with human disease phenotypes. The first break came with the finding that ABCB4 mediates the transport of phosphatidylcholine across the canalicular membrane during bile formation, and mutations in the *ABCB4* gene are a cause of progressive familial intrahepatic cholestasis.[33–35] Second, ABCA4 (ABCR) was suggested to transport a complex of retinaldehyde and phosphatidylethanolamine in the retina of the eye, and malfunctioning of this transporter results in Stargardt's macular dystrophy[36] and is related to age-related macular degeneration.[37] Third, half-size peroxisomal ABC proteins (ALDP, ALDR, PMP70, P70R) are suggested to be involved in the transport of long chain and very long chain fatty acids into peroxisomes.[38,39] Fourth, ABCB11 was proved to function as an ATP-dependent transporter of bile salt, which is converted from cholesterol in liver.[40] Finally, mutations in the *ABCA1* gene were causatively linked to familial high-density lipoprotein deficiency and Tangier disease.[41–45] ABCA1 plays a key role in the formation of HDL particles.[46–48] ABCG5 and ABCG8 are involved in the secretion of plant sterols and cholesterol into bile, and mutations in either of these genes are a cause of sitosterolemia.[49] ABCA7 also mediates apoA-I-dependent release of cholesterol and phospholipids.[50,51]

ABCA1 and HDL formation

Dietary cholesterol is absorbed from the small intestine and transported as chylomicron to the liver, from where it is