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Estrogen-induced regulation of the ATP-binding cassette transporter A1 (ABCA1) in mice: A possible mechanism of atheroprotection by estrogen

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Abstract

Estrogens are suggested to be antiatherogenic by affecting the vessel wall components. Since ABCA1 was recently shown to be atheroprotective, it was examined if estrogen-induced atheroprotection occurs partly via the regulation of the ABCA1. Since hepatic ABCA1 expression was also suggested to contribute to the bulk HDL levels, regulation of the ABCA1 under conditions of high or low levels of HDL were investigated in mice expressing normal or elevated levels of apoAI. To delineate whether estrogen's effect occurs via estrogen receptor-α-mediated pathway, the estrogen receptor-α-deficient $(ER\text{-}\alpha)^{-/-}$ mice were also administered either placebo or β-estradiol for 5 consecutive days. Estrogen treatments decreased circulating HDL levels by 30%, but increased hepatic and intestinal ABCA1 mRNA by 2- and 1.5-fold, respectively. Hepatic ABCA1 mRNA also increased in the $ER\text{-}\alpha^{-/-}$ mice by 3-fold. These results suggest that estrogen, despite lowering the levels of HDL, it up-regulated the hepatic ABCA1 mRNA, and in the absence of ER-α, ER-β could compensate for ER-α. To study whether HDL levels correlate with the ABCA1 expression, wild-type (WT) and the apoAI transgenic (A1-Tg) mice were fed high fat (HF) diet with or without cholic acid (CA) for 3 weeks. One group of mice was treated with fenofibrate, known to elevate HDL levels. CA without HF decreased HDL levels, while fenofibrate increased HDL levels. However, neither CA nor fenofibrate altered hepatic ABCA1 mRNA levels. HF diet increased the hepatic ABCA1 mRNA 1.8-fold in WT, but lowered ABCA1 mRNA by 2-fold in A1-Tg mice, suggesting that ABCA1 levels did not correlate with circulating HDL levels, while basal levels of HDL influenced ABCA1 expression. These data show for the first time that estrogen's antiatherogenic effects may occur via ABCA1-mediated pathway, and circulating HDL levels may influence expression of ABCA1. (Mol Cell Biochem **240**: 67–73, 2002)

Key words: estrogen, ABCA1, atherosclerosis, apoA1 transgenic mice, high fat

Abbreviations: ABCA1– ATP-binding cassette transporter A1; ER – estrogen receptor; CA – cholic acid; HF – high fat; PCR – polymerase chain reaction; cAMP – cyclic AMP; apo – apolipoprotein; Tg – transgenic; WT – wild-type

Introduction

ATP-binding cassette transporter A1 (ABCA1) was recently identified as the mutant protein responsible for the Tangier disease (TD) with incredibly low levels of HDL cholesterol, tissue cholesterol ester accumulation, and susceptibility to cardiovascular disease [1–3]. ABCA1 participates in the facilitation of cholesterol efflux from tissues to the acceptor lipid-poor apoA1 particles resulting in the formation of spherical mature cholesterol ester-rich HDL particles [4–6]. This mature HDL carries cholesterol to the liver for excretion as bile in a process called reverse cholesterol transport. As evidenced by the mutations in the TD and in the ABCA1-knockout mice [7, 8], any defect in this protein causes impaired cholesterol efflux from tissues to the nascent discoidal HDL particles, and leads to the deficiency of mature HDL.

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Further studies with ABCA1 transgenic mice [9, 10] suggested that over-expression of ABCA1 increases the plasma levels of HDL. This was also supported by the findings in heterozygote humans with mutation in ABCA1 that the levels of HDL correlated with the cholesterol efflux in the fibroblast [11]. Recent studies suggested that by inducing ABCA1, it is possible to inhibit the progression of atherosclerosis [12–14]. Taken together, ABCA1 appears to be an important player in inhibiting the progression of atherosclerosis by removing cholesterol from the lipid-laden arterial walls.

Estrogen's antiatherogenic properties, in the absence of lipoprotein modulation, appear to occur via interaction with the components of the vessel walls and modulation of some of the components at the site of the initiation of atherogenesis. Since ABCA1 is associated with the inhibition of atherogenesis, it was hypothesized that some of the antiatherogenic activities of estrogen occurs via ABCA1-mediated pathway. Estrogen has been clearly shown in apoE knockout mice to inhibit the progression of atherosclerosis [15]. Among other factors, the atheroprotective effect of estrogen was suggested to occur via modulation of the apoE gene expression [16]. The upregulation of the apoE gene expression, and the inhibition of atherogenesis were found to occur via estrogen receptor-α (ER-α)-mediated pathway [16, 17]. Given the roles of ABCA1 in the athero-protection, and the roles of ER-α in the estrogen-mediated antiatherogenic effects, experiments were carried out to examine whether estrogen-induced antiatherogenic activities occur via ABCA1-mediated pathway, and whether ER-α receptor is required in this process.

Based on the recent studies, a consensus is building that the expression of ABCA1 levels correlate with the circulating levels of HDL. Although two studies with the ABCA1 transgenic mouse lines showed a correlation between ABCA1 expression and plasma levels of HDL, no correlation was found in another study [18], raising the possibility that ABCA1 expression may not always correlate with the plasma levels of HDL. In a recent study [19], macrophage expression of the ABCA1, although correlates with the cholesterol efflux, contributed very little to the bulk HDL in circulation, suggesting liver ABCA1 expression as the major contributor to the plasma levels of HDL. Whether or not HDL levels indeed correlate with the hepatic levels of ABCA1 has been addressed in the present study using wild-type mouse with basal levels of HDL, and apoA1-transgenic mouse line with elevated levels of HDL in two conditions that suppress [20] or elevate [21] the plasma levels of HDL. Based on the results of the present study, it is shown for the first time that one of the mechanisms of estrogen-mediated inhibition of progression of atherosclerosis may occur via ABCA1-mediated pathway, and that modulation of the HDL levels may not always correlate with the ABCA1 expression.

Materials and methods

Estrogen treatments

Male C57BL/6J mice were procured from The Jackson Laboratories, Bar Harbor, ME, USA. These mice were allowed to acclimatize for one week on chow diet after arrival, and divided into two groups of 5 mice each. The placebo group received vehicles only. The treatment group was administered 17β-estradiol at 3 μg/g body wt/day for 5 consecutive days by subcutaneous implantation (Innovative Research of America, Toledo, OH, USA). The estrogen receptor-α knock out mouse line has been described before [16]. These mice had a mixed background of 129/J and C57BL/6J. To eliminate any potential strain background effects, wild-type littermates were used as control group. These mice were also divided into two groups and treated the same way as the C57BL mice. On the sixth day, mice were fasted for 4–6 h and analyses were made.

Feeding experiment

ApoA1 transgenic mice prepared using the 11 kb genomic fragment containing natural promoter developed by Edward M. Rubin, Berkley Laboratories, CA, USA were obtained from Jackson Laboratories, Bar Harbor, ME, USA. The wild-type littermates and the transgenic mice were divided into four groups as follows: control (C) – chow with 5% corn oil; chow with cholic acid (CA) – 1% cholic acid; chow with high fat (HF) – 20% hydrogenated coconut oil plus 0.5% cholesterol; chow with high fat plus cholic acid (HF + CA) – HF plus 1% cholic acid. WT – littermates and the apoA1 transgenic mice were fed these diets *ad libitum* for 3 weeks. In another experiment, apoA1 transgenic mice were fed fenofibrate (0.5% mixed with chow) for 1 week as described [21]. At the end of the feeding experiment, mice were fasted for 4–6 h, sacrificed, and necessary analyses were made.

Lipoprotein and apoprotein analysis

Plasma lipoprotein analysis was done by gel permeation chromatography as described [22, 23]. In each fraction, cholesterol, triglycerides and phospholipids were quantitated by enzymic methods. HDL levels were measured as described [24]. Plasma apoA1 levels were quantitated by ELISA assay [20].

Preparation and analysis of RNA

RNA was prepared from fresh liver and small intestines using the one step RNA isolation method procured from CloneGen

Biotechnology, Ann Arbor, MI, USA [25]. The integrity of the RNA was determined by performing Northern blotting analysis using the reagents and riboprobe obtained from CloneGen Biotechnology, Ann Arbor, MI, USA [26]. Human and mouse apoA1 mRNA were quantified by RNAse protection assays using the linearized recombinant plasmid procured from CloneGen Biotechnology [26]. The ABCA1 mRNA was quantitated by real-time RT-PCR using Taqman 7900HT machine, (ABI Prism) [9]. 18S RNA was used as an internal control. The probe and primer for ABCA1 quantitation were designed by PrimerExpress software. Sixty nanogram total RNA was taken in triplicate from each mouse (N = 4) to quantitate ABCA1 mRNA.

Cholesterol efflux assay

Cholesterol efflux assay was done in J774 cells following the standard method [28]. Cyclic-AMP was used as a positive control, since it has been shown to enhance cholesterol efflux in the presence of apoA1 [29].

Results

Effect of estrogen administration on the ABCA1 *gene expression*

Effect of estrogen on the regulation of the *ABCA1* gene expression and circulating levels of HDL was studied in C57BL/6J mice administered β-estradiol for 5 consecutive days. As shown in Fig. 1A mice administered estradiol decreased HDL levels by 30% with concomitant decreases in the plasma levels of apoA1. Since hepatic levels of apoA1 mRNA increased by estradiol treatment, the lowering of plasma levels of HDL may have not occurred via transcriptional mechanism. Hepatic ABCA1 mRNA was quantitated by a sensitive Taqman realtime PCR, and the results are shown in Fig. 1B. Estradiol treatment increased the hepatic and intestinal levels of ABCA1 mRNA by 1.8- and 1.5-fold, respectively. Since estrogen receptor (ER)-α is the major estrogen receptor responsible for the estrogen-mediated gene regulation, effect of estradiol administration was studied in ER-$\alpha^{-/-}$ mice. Estrogen administration increased hepatic ABCA1 mRNA even in the ER-deficient mice, suggesting that in the absence of ER-α, other receptor, possibly ER-β, becomes the major player in the estrogen-mediated regulation of the ABCA1.

Effect of cholic acid, high fat, and fenofibrate treatments on ABCA1

To study the regulation of the hepatic ABCA1 by dietary fat (HF), cholic acid (CA), and fenofibrate, wild-type (WT) and

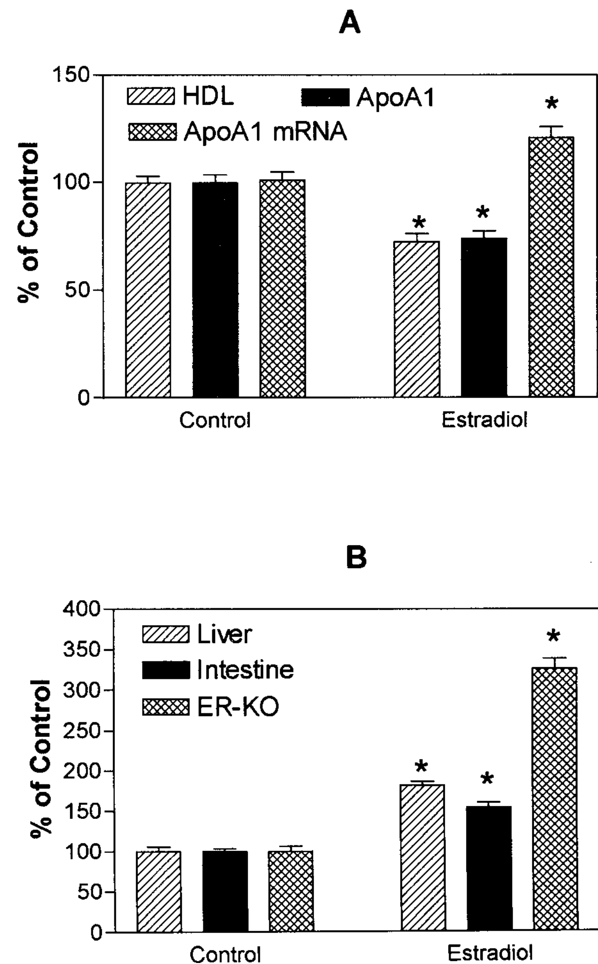

Fig. 1. Panel A: Plasma levels of HDL and apoA1, and hepatic levels of apoA1 mRNA in mice treated with β-estradiol for 5 consecutive days. The values in the control group have been assigned 100, and the values in the treated groups are percent of control. *Significantly different when compared to control group (N = 5). Panel B: Hepatic levels of ABCA1 in WT and the estrogen receptor-α knockout mice quantitated by Taqman 7900HT using mouse-specific probe and primers. *Significantly different compared to control group.

the apoA1 transgenic (A1-Tg) mice were fed control, cholic acid, high fat, and high fat plus cholic acid diets. In another experiment, apoA1 transgenic mice with high plasma levels of HDL and apoA1, were treated with fenofibrate to further elevate plasma levels of HDL and study the ABCA1 regulation under the conditions of high and low levels of HDL. The results of dietary fat and CA feeding in WT and the A1-Tg mice are shown in Fig. 2. CA feeding to WT mice lowered plasma levels of HDL and apoA1 by 40%, while high fat feeding elevated plasma levels of HDL and apoA1 by 60%, when compared to control. Supplementation of CA to HF diet suppressed plasma levels of HDL (Fig. 2A). In the A1-Tg mice, the effect CA was more pronounced (Fig. 2B). The plasma levels of HDL and apoA1 decreased by more than 2.5-

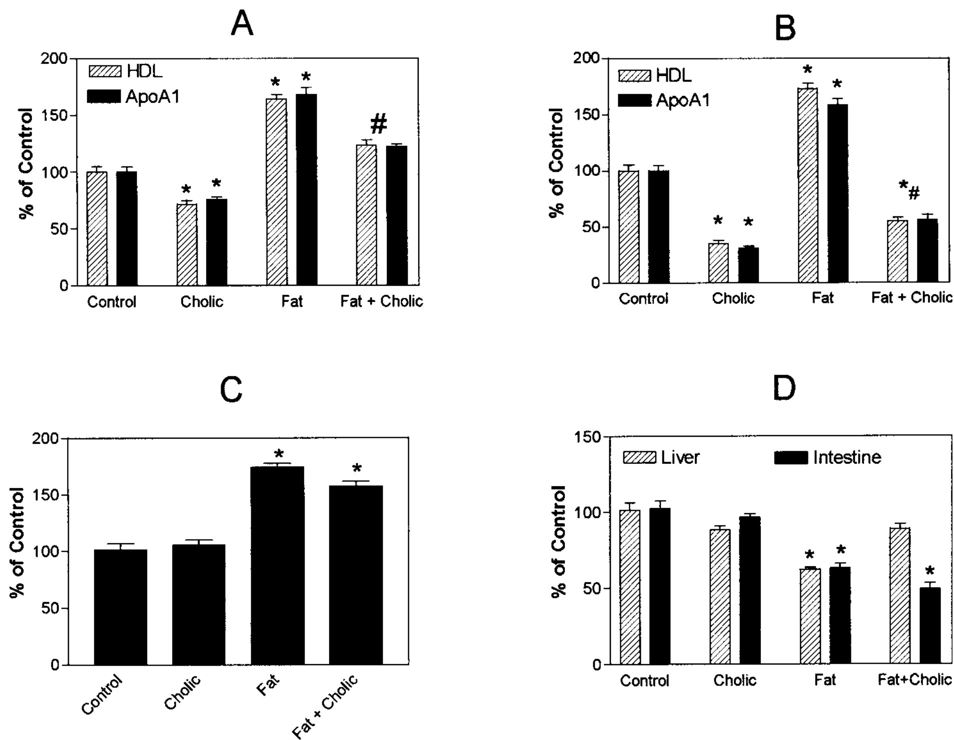

Fig. 2. Plasma levels of HDL and apoA1 in WT (panel A) and apoA1 transgenic mice (panel B) fed control, cholic acid, high fat and high fat plus cholic acid diet for 3 weeks. Plasma levels of HDL and apoA1 were measured by standard method. Panels C (WT) and D (apoA1-transgenic) show quantitation of ABCA1 mRNA in the hepatic RNA from WT mice, and hepatic and intestinal RNA from apoA1 transgenic mice. *Significantly different when compared to control. #Significantly different compared to high fat diet.

fold following CA feeding in A1-Tg mice, while high fat feeding increased HDL and apoA1 levels by 65%. Addition of CA to high fat diet lowered plasma levels of HDL and apoA1.

Hepatic ABCA1 mRNA was quantitated in mice fed various diets or treated with fenofibrate. In WT mice, CA feeding did not influence hepatic levels of ABCA1, while high fat feeding elevated hepatic levels of ABCA1 mRNA by 1.8-fold (Fig. 2C). Supplementation of high fat diet with CA also increased hepatic levels of ABCA1 mRNA by 1.6-fold, suggesting that high fat diet elevated hepatic levels of ABCA1 mRNA in the presence or absence of CA. When A1-Tg mice were treated the same way and ABCA1 mRNA quantitated, surprisingly, hepatic ABCA1 mRNA decreased by 2-fold in HF-fed mice, and cholic acid supplementation of high fat diet abolished the effect of high fat diet on ABCA1 mRNA (Fig. 2D). However, intestinal ABCA1 mRNA decreased by 2-fold in HF-fed and HF plus CA-fed mice. These results suggested that basal levels of HDL influence the hepatic levels of ABCA1. To further study whether elevation of plasma levels of HDL has any influence on the regulation of hepatic ABCA1 mRNA, the A1-Tg mice with higher basal levels of HDL were treated with fenofibrate, resulting elevation of plasma levels of HDL by 5-fold (Fig. 3). Quantitation of hepatic ABCA1 mRNA in fenofibrate treated mice showed no changes in the levels of ABCA1 mRNA despite very high levels of circulating HDL. Since CA lowered plasma levels of HDL and apoA1 by more than 2-fold, but did not influence hepatic levels of ABCA1 mRNA, it was hypothesized

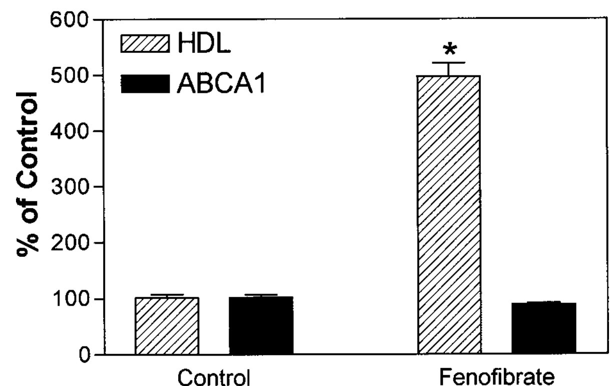

Fig. 3. Plasma levels of HDL and hepatic levels of ABCA1 mRNA in apoA1 transgenic mice fed either chow diet or 0.5% fenofibrate plus chow as diet admixture for one week as described [21]. *Significantly different when compared to control.

that CA may have minimal effects on cholesterol efflux. To test this hypothesis, J774 cells were loaded with labeled cholesterol and efflux of cholesterol measured in the presence and absence of CA. Cholic acid did not influence cholesterol efflux in J774 cells (data not shown).

Discussion

The aim of the present study was to examine if estrogen's atheroprotective effects, among other pathways, may also occur via ABCA1-mediated pathway. Indeed, estrogen treatment increased ABCA1 mRNA levels by ~ 2-fold, suggesting that among other mechanisms, estrogen may also impart cardioprotection via ABCA1-mediated pathway. Since estrogen also increases plasma levels of apoE in mice [16], which is involved in cholesterol efflux [29], this may have partly contributed to the estrogen-mediated atheroprotection. Since estrogen's ability to raise plasma levels of apoE was abolished in ER-α-deficient mice [16], therefore, experiments were also carried out in the ER-α-deficient mice to examine if estrogen's ability to upregulate ABCA1 mRNA is dependent on the ER-α. Interestingly, estrogen increased hepatic ABCA1 mRNA even in the ER-α-deficient mice, suggesting that the presence of ER-α is not a prerequisite for estrogen-mediated regulation of the hepatic ABCA1, and other estrogen receptors, like ER-β, may replace the function of ER-α [31]. In the double knock out mice, $apoE^{-/-}/ER\text{-}\alpha^{-/-}$, estradiol treatment reduced lesion size, but not to the same extent as in the apoE-deficient mice, suggesting that part of the estrogen-mediated atheroprotection possibly occur independent of ER-α. Therefore, it is not surprising that estrogen increased the hepatic ABCA1 expression in the absence of ER-α. That the atheroprotective effects of estrogen occur largely via lipoprotein-independent mechanism was further supported by the observations that estrogen treatment decreased HDL in mice via hepatic lipase-mediated pathway [32]. How could estrogen then have atheroprotection when it lowers plasma levels of HDL? It should be noted that not only the HDL levels, but the functional HDL pool is important in removing arterial cholesterol. It is possible that the decreased levels of HDL in the estrogen treated mice resulted from faster turnover of HDL particles, since larger HDL particles are catabolized at a slower rates compared to the smaller HDL particles [33], and more delivery of cholesterol to the liver may increase ABCA1 [34]. Given the increased expression of macrophage ABCA1 mRNA when hepatic ABCA1 levels are stimulated [9, 10], it is likely that the estrogen-mediated up-regulation of the hepatic ABCA1 expression also paralleled increased ABCA1 mRNA in macrophages [14]. Thus, estrogen-mediated upregulation of the ABCA1 expression may contribute to the accelerated removal of cholesterol from the peripheral tissues, and inhibit the progression of atherosclerosis.

An important finding in the present study is the estrogen-mediated lowering of plasma levels of HDL, despite up-regulation of hepatic ABCA1 mRNA. Over-expression of ABCA1 elevates circulating HDL levels [9, 10], and individuals heterozygous for the ABCA1-null mutation showed a correlation between plasma levels of HDL and ABCA1-mediated cholesterol efflux in fibroblasts [11]. A recent study also demonstrated that the hepatic, but not the macrophage ABCA1 expression, is important in determining the plasma levels of HDL [19]. Taken together, these studies demonstrate that the hepatic expression of ABCA1 is important in determining the plasma levels of HDL. However, this correlation was absent in the estrogen-treated mice. The up-regulation of the hepatic ABCA1 mRNA may be associated either with the cellular cholesterol levels that regulate ABCA1 expression, or increased secretion of apoE [16], which may influence the ABCA1 regulation. In a recent study, the ABCA1 and the apoE secretions increased in parallel following treatment of macrophages with cAMP [35].

The second aim of the present study was to examine if modulation of plasma levels of HDL can also modulate hepatic ABCA1 expression, since increased hepatic ABCA1 mRNA was not associated with the plasma levels of HDL in estrogen-treated mice. Feeding high fat diet increased plasma levels of HDL and hepatic ABCA1 mRNA in WT mice. However, feeding cholic acid, although lowered HDL levels, it did not alter hepatic levels of ABCA1 mRNA, suggesting that under certain conditions circulating HDL levels may not correlate with the hepatic levels of the ABCA1. High fat diet raised HDL levels concomitant with increased levels of the hepatic ABCA1 mRNA. To further corroborate the lack of correlation between circulating HDL levels and the hepatic ABCA1 mRNA, experiments were carried out in the apoA1 transgenic mice with high basal levels of HDL. In A1-Tg, cholic acid lowered plasma levels of HDL dramatically without altering hepatic ABCA1 mRNA. High fat diet increased plasma levels of HDL by 65%, but hepatic ABCA1 mRNA instead of showing increases, decreased by almost 2-fold. Intestinal ABCA1 mRNA also decreased on high fat diet. These findings further support the hypothesis that circulating HDL levels may not always correlate with the hepatic ABCA1 mRNA levels. Since circulating levels of HDL in A1-Tg mice are already very high, the reverse cholesterol transport in these mice occurs at an accelerated rate, resulting in the increased delivery of cholesterol to the liver, and modulation of ABCA1 mRNA. Indeed, the hepatic ABCA1 mRNA was higher (1.6-fold) in A1-Tg mice compared to WT mice. ABCA1 is a high efficiency- low capacity molecule in the cholesterol efflux process. Since ABCA1 is already in an induced state in the A1-Tg mice, further elevation of HDL by high fat diet possibly down-regulates hepatic ABCA1. Fenofibrate has been earlier shown to up-regulate ABCA1

gene expression in macrophages [36], but it is not known whether fenofibrate regulates the hepatic ABCA1 mRNA. Treating A1-Tg mice with fenofibrate increased plasma levels of HDL by 500%, yet no significant changes observed in the levels of hepatic ABCA1 mRNA.

In summary, we show that estrogen administration to mice increased hepatic levels of ABCA1. These studies suggest that the atheroprotective effects of estrogens may occur, in part, via ABCA1-mediated pathway. We also show that circulating HDL levels may not always correlate with the hepatic ABCA1 expression.

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