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Overexpression of the steroidogenic acute regulatory protein increases the expression of ATP-binding cassette transporters in microvascular endothelial cells (bEnd.3)*

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Abstract: Objective: To determine the effect of steroidogenic acute regulatory protein (StAR) overexpression on the levels of adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1) in an endothelial cell line (bEnd.3). Methods: The StAR gene was induced in bEnd.3 cells with adenovirus infection. The infection efficiency was detected by fluorescence activated cell sorter (FACS) and fluorescence microscopy. The expressions of StAR gene and protein levels were detected by real-time polymerase chain reaction (PCR) and Western blot. The gene and protein levels of ABCA1 and ABCG1 were detected by real-time PCR and Western blot after StAR overexpression. Results: The result shows that StAR was successfully overexpressed in bEnd.3 cells by adenovirus infection. The mRNA and protein expressions of ABCA1 and ABCG1 were greatly increased by StAR overexpression in bEnd.3 cells. Conclusion: Overexpression of StAR increases ABCA1 and ABCG1 expressions in endothelial cells.

Key words: Steroidogenic acute regulatory protein (StAR), Endothelial cells, Cholesterol, Adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1), bEnd.3
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1 Introduction

Hypercholesterolemia is the most important risk factor for atherosclerosis and other cardiovascular diseases. Maintenance of cholesterol homeostasis is an effective protective mode against the development of atherosclerosis. Endothelial cells (ECs) are one of the three kinds of cells involved in the formation of atherosclerotic plaque. Other cells involved, such as macrophages, form foam cells, which contain low-density lipoprotein (LDL) compounds. ECs will not, however, form foam cells even though they express the receptors for LDL or oxidized LDL (ox-LDL) (Hassan *et al*., 2006; O'Connell *et al*., 2004). Cholesterol homeostasis in ECs remains poorly understood. Understanding the mechanisms by which vascular ECs maintain tight cholesterol homeostasis may shed light on the mechanism of cellular sterol transport.

High density lipoprotein (HDL)-dependent reverse cholesterol transport (RCT) is thought to be the most important mode for reducing cholesterol levels in peripheral cells. Studies have indicated that adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1) is one of the cholesterol transporters which transfer cholesterol from cytoplasm to apoprotein A-I (apoA-I) to form HDL, and is crucial in the RCT from peripheral tissues, such as ECs (Brewer

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and Santamarina-Fojo, 2003). Another study has shown that another transporter, ATP-binding cassette transporter G1 (ABCG1), is highly induced in cholesterol-loaded human ECs (O'Connell *et al*., 2004).

Steroidogenic acute regulatory protein (StAR/StarD1) is one of the cholesterol transporters in steroidogenic tissues, and can transfer cholesterol to the inner mitochondria for cholesterol oxidation at the site where sterol 27-hydroxylase (CYP27A1) is located. This step is rate-limiting in steroidogenesis (Christenson and Strauss III, 2000; Stocco, 2000). StAR is also expressed in the liver, with a similar function (Hall *et al*., 2005). Pandak *et al*. (2002) proved that cholesterol transport to the inner mitochondrial membrane by StAR was the rate-limiting step for bile acid synthesis through the alternative pathway initiated by CYP27A1. In the mitochondria, cholesterol can be oxidized by CYP27A1 into 27-hydroxycholesterol or other soluble products.

In our previous work, we have found that pro-inflammatory cytokines, such as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), can downregulate the expression of StAR while anti-inflammatory factor transform growth factor beta-1 (TGFβ1) can upregulate it in several kinds of macrophages (Ma *et al*., 2007). Overexpression of StAR in human acute monocytic leukemia cell line (THP-1)-derived macrophages decreases the cellular lipid levels by upregulating the levels of ABCG1 and liver-X receptor alpha (LXRα) (Ning *et al*., 2009). The role of StAR in cholesterol metabolism in ECs, however, is still not well understood. Previous reports have shown that mRNA and protein levels of StAR are highly regulated by cholesterol, LDL, and 25-hydroxycholesterol in mouse brain microvascular EC line (bEnd.3) (Ning *et al*., 2006). In this study, we overexpressed StAR gene in bEnd.3 cells by adenovirus transfection, then examined the levels of ABCA1 and ABCG1 mRNA and protein following StAR overexpression.

2 Materials and methods

2.1 Reagents

All cell culture reagents, TRIzol and SuperScript™ III first-strand synthesis system were purchased from Invitrogen Life Technologies Inc. (Grand Island, NY). PageRuler™ prestained protein ladder was purchased from Fermentas MBI (San Diego, CA). SYBR® green real-time polymerase chain reaction (PCR) master mix was obtained from Toyobo Company (Osaka, JP). Primary antibodies against StAR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Abcam Ltd. (Cambridge Science Park, Cambridge, UK). ABCG1 and ABCA1 antibodies were purchased from Santa Cruz (Santa Cruz, CA). Second antibodies were purchased from Kirkegaard & Perry Laboratories (Guildford, UK). Enhanced chemiluminescent (ECL) reagent for bands detection was purchased from Pierce Biotechnology, Inc. (Rockford, IL).

2.2 Cell culture

Mouse brain microvascular EC line, bEnd.3, received from American Type Culture Collection (ATCC, Manassas, VA), was cultured as previously described (Ning *et al*., 2006). Its cytological characteristics were detected by cluster of differentiation 34 (CD34) and von Willebrand factor (vWF) (Yue *et al*., 2004).

2.3 Cell infected by adenovirus encoding StAR

bEnd.3 cells were transfected by recombinant adenovirus encoding StAR (Ad-CMV-StAR) at a multiplicity of infection (MOI)=10 plaque-forming units (PFUs)/cell for 48 h, as previously described (Ning *et al*., 2009). Cells infected by control adenovirus encoding enhanced green-fluorescence protein infections (Ad-CMV-EGFP) and cells without virus addition (Con.) acted as controls for all experiments.

2.4 Quantitive real-time reverse transcription PCR

Real-time reverse transcription PCR was done as previously described (Ning *et al*., 2006). GAPDH was used as an internal control. The mRNA expressions of StAR, ABCA1 and ABCG1 were detected in bEnd.3 cells after infection. The sequences of the primers used in this experiment are shown in Table 1.

2.5 Western blot analysis

After infected by adenovirus, cells were lysed in radioimmune precipitation assay (RIPA) buffer, or subcellular parts were separated and harvested as previously described (Hall *et al*., 2001). Thirty micrograms of total proteins were loaded to detect

Table 1 Sequences of primers used in the experiment

Gene	Gene ID	Primer sense	Primer antisense
StAR	S79669	TCTCTACTCGGTTCTCGG	TCCTGCTGACTCTCCTTC
ABCA1	NM_013454	TGGTTTGTTAGCAGCCTCATC	AGCACTGTAGGATGGTCACC
ABCG1	NM_009593	GTACCATGACATCGCTGGTG	AGCCGTAGATGGACAGGATG
GAPDH	BC083149	ACAGCCGCATCTTCTTGTGCAGTG	GGCCTTGACTGTGCCGTTGAATTT

ABCG1 or ABCA1, using GAPDH as a loading control. Twenty micrograms of mitochondrial proteins were loaded for StAR determination. Western blot was done as previously described (Ning *et al*., 2009).

2.6 Statistics

Data were described as mean±standard deviation (SD), and further subjected to analysis of variance (ANOVA). For all analyses, an alpha value of <0.05 was considered significant.

3 Results

3.1 Infection of bEnd.3 cells by adenovirus

The bEnd.3 cells were infected by Ad-CMV-StAR or Ad-CMV-EGFP, respectively. Forty-eight hours after infection, the infection efficiency was analyzed by fluorescence activated cell sorter (FACS). Fig. 1b shows that the infection efficiency was about 96.09%. The GFP-expressing in cells were photographed by fluorescence microscope (Figs. 1c and 1d).

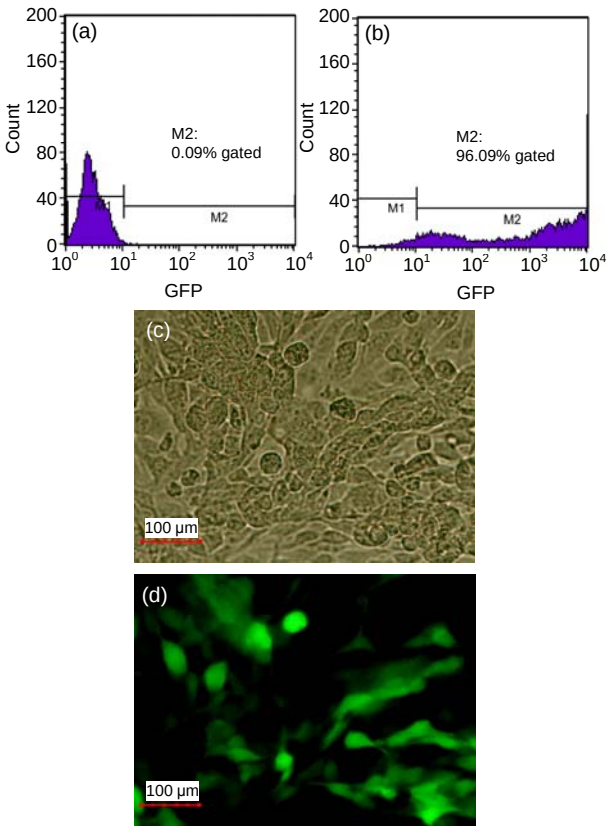

Fig. 1 bEnd.3 cells were successfully infected by adenovirus
(a) & (b) Infection efficiency detected by FACS. Ten thousand bEnd.3 cells were gated and the percentage of fluorescent cells (% gated) was used to show infection efficiency. (a) Untreated bEnd.3 cells; (b) Ad-CMV-EGFP-infected bEnd.3 cells. About 96.09% cells expressed eGFP after 48 h infection. (c) & (d) Fluorescence microscopic images of bEnd.3 cells which were infected by adenovirus. (c) Infected cells imaged under bright light; (d) The same cells population imaged by fluorescence microscope

3.2 Expressions of StAR mRNA and protein levels in bEnd.3 cells following adenovirus infection

In order to determine whether StAR was overexpressed in bEnd.3 cells after adenovirus infection, we detected the mRNA and protein levels of StAR in the cells by real-time RT-PCR and Western blot. As seen in Fig. 2, StAR mRNA and protein levels were increased about 160- and 50-fold, respectively, compared to the no-virus and control-virus groups.

3.3 Expressions of ABCA1 and ABCG1 mRNA and protein levels following StAR overexpression in bEnd.3 cells

In previous work, we demonstrated StAR expression in bEnd.3 cells for the first time (Ning *et al*., 2006). The effect of StAR on cholesterol transporter expression in the ECs, however, was unclear. To test whether StAR could affect the cholesterol homeostasis in ECs, we investigated the effects of StAR overexpression on the expressions of cholesterol transporters ABCA1 and ABCG1.

bEnd.3 cells were infected by adenovirus encoding StAR for 48 h before being harvested for mRNA and protein assays. As Figs. 3a and 3b show, ABCA1 mRNA and protein expressions were increased by 2- and 3-fold, respectively, following adenovirus infection. In addition to ABCA1, ABCG1 is another important cholesterol transporter in ECs.

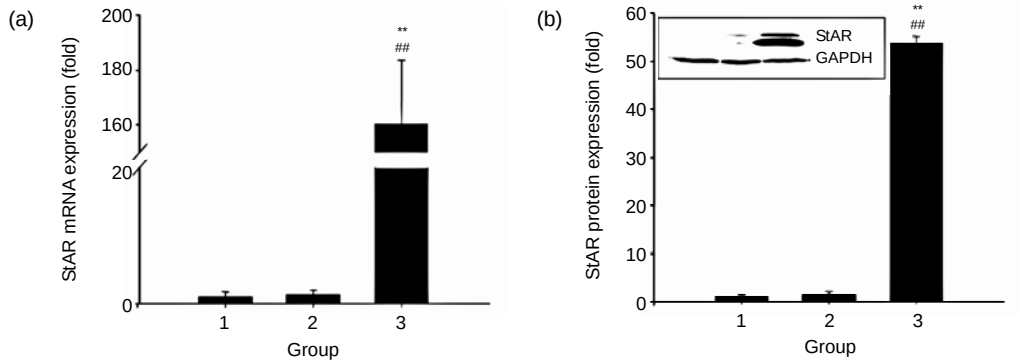

Fig. 2 StAR mRNA and protein expression levels were greatly increased following adenovirus infection in bEnd.3 cells

(a) StAR mRNA levels, after infected with adenovirus, were detected by real-time PCR. The experiments were repeated three times with similar results. Data were normalized to GAPDH mRNA and represented as the orders of magnitude higher than that of no-virus control (Con.); (b) StAR protein expression, after infection with adenovirus, was detected by Western blot. Data were normalized to GAPDH protein after being quantified by phosphor imaging. The upper panel shows the bands of StAR protein. Statistical results from three separate experiments are represented in the lower panel. Data were represented as the orders of magnitude higher than that of no-virus control (Con.). Group: 1: Con., cells without virus; 2: Ad-CMV-EGFP, cells transfected with control adenovirus with CMV-EGFP; 3: Ad-CMV-StAR, cells transfected with adenovirus with CMV-StAR. ** $P<0.01$ vs. Con., $^{\#\#}$ $P<0.01$ vs. Ad-CMV-EGFP

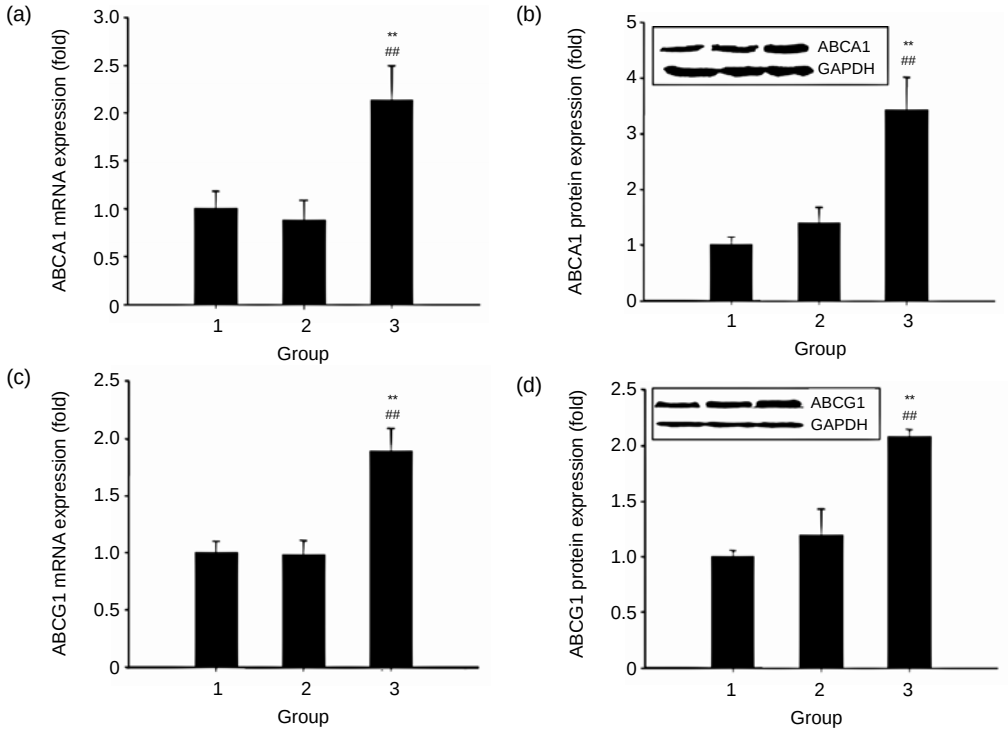

Fig. 3 ABCA1 and ABCG1 mRNA and protein expressions were increased following StAR overexpression in bEnd.3 cells

ABCA1 mRNA (a) and ABCG1 mRNA (c) levels after infection with adenovirus were determined by real-time PCR. The experiments were repeated three times with parallel results. Data were standardized to GAPDH mRNA and represented as the orders of magnitude higher than that of no-virus control (Con.). ABCA1 protein (b) and ABCG1 protein (d) expressions after infection with adenovirus were determined by Western blot. Data were standardized to GAPDH levels after being quantified by phosphor imaging. The upper panel indicates the bands of protein. Statistical data from three separate experiments were represented in the lower panel. Data were represented as the order of magnitude higher than that of no-virus control (Con.). Group: 1: Con., cells without virus; 2: Ad-CMV-EGFP, cells transfected by control adenovirus with CMV-EGFP; 3: Ad-CMV-StAR, cells transfected by adenovirus with CMV-StAR. ** $P<0.01$ vs. Con., $^{\#\#}$ $P<0.01$ vs. Ad-CMV-EGFP

Similar to ABCA1, mRNA and protein levels of ABCG1 were also increased about 2-fold in bEnd.3 cells following StAR overexpression (Figs. 3c and 3d).

4 Discussion

Cholesterol homeostasis in ECs remains unclear. Unlike smooth muscle cells and macrophages, which also form the atherosclerotic plaque, vascular ECs do not accumulate cholesterol. Cholesterol efflux pathways might play important roles in this process. Thus, studying the mechanism of cholesterol homeostasis in ECs assists in understanding the process of atherosclerosis and protection against it. O'Connell *et al*. (2004) found that the expression of ABCG1 was greatly increased in cholesterol-loaded human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs). ABCG1, as well as ABCA1, plays a vital role on HDL-dependent cholesterol efflux in rat aortic ECs (Lin *et al*., 2009). In the present study, we have shown that StAR overexpression can increase the mRNA and protein expression levels of ABCA1 and ABCG1 in microvascular ECs, bEnd.3 cells, indicating that StAR plays an essential role in the maintenance of cholesterol homeostasis in ECs, as well as in hepatocytes and macrophages, as previously reported (Hall *et al*., 2005; Ning *et al*., 2009).

ABCA1 leads to the efflux of cholesterol and phospholipids to apoA-I, and the subsequent formation of nascent HDL particles (Liu *et al*., 2003), while ABCG1 promotes cholesterol efflux from cells to HDL, rather than to apoA-I (Kennedy *et al*., 2005; Vaughan and Oram, 2005). As shown in previous work, both of these transporters are primarily regulated by LXRs (Venkateswaran *et al*., 2000). Oxysterols are physiological ligands for LXRs (Repa and Mangelsdorf, 2002). 27-hydroxycholesterol and 22-hydroxycholesterol produced by CYP27A1 can activate LXRs in cholesterol-loaded cells (Fu *et al*., 2001; Repa and Mangelsdorf, 2002). CYP27A1 activities have been demonstrated to play an essential role in the maintenance of cholesterol homeostasis in vitro and in vivo (Dubrac *et al*., 2005; Gueguen *et al*., 2007). Recent studies have indicated that StAR plays the rate-limiting role for CYP27A1 activities (Hall *et al*., 2005; Pandak *et al*., 2002; Sugawara *et al*., 1995). Thus, StAR may regulate cholesterol homeostasis through generating regulatory oxysterols and subsequently activating LXRs.

Previous studies have shown that CYP27A1 is essential in the clearance of cholesterol from peripheral tissues via transfer of cholesterol to the more soluble 27-hydroxycholesterol and 3-hydroxy-5-cholestenoic acid. Javitt (1994) indicated that there may be an efflux of 27-hydroxycholesterol from ECs to the liver. Babiker *et al*. (1997) showed that CYP27A1 can clear away cholesterol in ECs and macrophages. Hall *et al*. (2001) suggested that CYP27A1 might work as an antiatherogenic enzyme by synchronously facilitating the clearance of cholesterol from extrahepatic tissues and inhibiting cholesterol synthesis. Overexpression of StAR can upregulate the activity of CYP27A1 to engender regulatory oxysterols, this representing the ability to modulating intracellular cholesterol homeostasis (Hall *et al*., 2005). Other studies have indicated that overexpression of StAR in primary human and rat hepatocytes greatly increases regulatory oxysterols in nuclei. These regulatory oxysterols are powerful modulators that participate in cellular cholesterol metabolism (Li *et al*., 2007; Ren *et al*., 2006; 2007). Our work in macrophages has shown that overexpression of StAR can decrease the cellular lipid levels via enhancing the levels of CYP27A1 and ABCG1 (Ning *et al*., 2009). The levels of StAR can be regulated by cholesterol, LDL and 25-hydroxycholesterol in bEnd.3 cells, which indicates that the StAR expression in these cells has a physiological significance. All of this evidence supports that StAR plays an essential role in cholesterol homeostasis in ECs through stimulating CYP27A1 activities.

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