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Research Paper

High glucose decreases the expression of ATP-binding cassette transporter G1 in human vascular smooth muscle cells☆

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Abstract

Objective:ATP-binding cassette transporters(ABC) A1 and G1 play an important role in mediating cholesterol efflux and preventing macrophage foam cell formation. In this study, we examined the regulation of ABC transporters by high glucose in human vascular smooth muscle cells(VSMCs), the other precursor of foam cells. **Methods:**Incubation of human VSMCs with D-glucose(5 to 30 mM) for 1 to 7 days in the presence or absence of antioxidant and nuclear factor(NF)-κB inhibitors, the expressions of ABCA1 and ABCG1 were analyzed by real time PCR and Western blotting. **Results:**High glucose decreased ABCG1 mRNA and protein expression in cultured VSMCs, whereas the expression of ABCA1 was not significantly decreased. Down-regulation of ABCG1 mRNA expression by high glucose was abolished by antioxidant N-acetyl-L-cysteine(NAC) and NF-κB inhibitors, BAY 11-7085 and tosyl-phenylalanine chloromethyl-ketone(TPCK). **Conclusion:**High glucose suppresses the expression of ABCG1 in VSMCs, which is the possible mechanism of VSMC derived foam cell transformation.

Key words: vascular smooth muscle cells(VSMCs); ABC transporters; glucose; oxidative stress; NF-κB pathway

INTRODUCTION

The prevalence, incidence, and mortality from all forms of atherosclerosis are increased in patients with diabetes[1]. However, molecular mechanisms underlying the increased rate of atherosclerosis in diabetic patients are poorly understood. Among the risk factors documented in diabetes, hyperglycemia appears as an independent risk factor for diabetic macrovascular complications[2, 3]. Possible links of hyperglycemia to atherosclerosis include an increase in oxidative stress, activation of protein kinase C(PKC) and NF-κB[3, 4], and then impairing components of atheromatus lesions, including vascular smooth muscle cells(VSMCs) and macrophages.

Members of the ABC transporter superfamily are known regulators of cholesterol efflux[5-9]. Of these, ABCA1 is considered as a key regulator of cholesterol and phospholipid export to lipid-free apoA-I, then forming nascent HDL[5, 6]. ABCG1, a half transporter member of the ABC superfamily, facilitates cholesterol efflux to HDL but not to lipid-free apoA-I[7, 8]. So ABCA1 and ABCG1 play an important role in reverse cholesterol transport, subsequently protecting against cellular lipid deposition and foam cell formation. Many metobolitic factors have been reported to regulate the expression of both ABCA1 and ABCG1 in macrophages, such as oxysterol and unsaturated fatty acid[6, 10]. Hyperglycemia also has been reported to decrease ABCG1 expression in diabetic mouse peritoneal macrophages(MPMs) and in glucose-cultured MPMs, thus leading to decreased cholesterol efflux and accelerated macrophage-derived foam cell production[11]. ABCA1 gene expression in peripheral leukocytes was shown to negatively associate with fasting glucose concentration in normoglycemic

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men[12]. VSMC is widely known as a precursor of foam cells. Whether ABC transporters in VSMC are altered as in macrophage is known to be limited. So we proceeded to investigate human VSMCs treated by D-glucose in vitro. The results showed that high glucose suppressed ABCG1, but not ABCA1 expression in cultured VSMCs. Furthermore, this effect was associated with increased oxidative stress and NF-κB activation induced by high glucose.

MATERIALS AND METHODS

Cell culture

Human umbilical artery smooth muscle cells (ScienCell, USA) were grown in Dulbecco's modified Eagle's medium(DMEM,Invitrogen,USA) supplemented with 10% fetal bovine serum(Gibco, USA), streptomycin(100 μg/ml), and penicillin G(100 U/ml) at 37℃ in a humidified atmosphere of 5% CO_2. Passages between 6 and 10 were used for the experiment. VSMCs were incubated for 1 to 7 days in DMEM medium containing 5 mM(normal glucose) to 30 mM D-glucose (high glucose) as well as pretreated for 1 hour with or without antioxidant NAC(10mM[3], Sigma, USA), the NF-κB inhibitors BAY 11-7085 (10 μM[13], Alexis Biochemicals, Switzerland) and TPCK(20 μM, Sigma, USA), and then exposed for 3 days to glucose of 5 mM or 30 mM. During the period of incubation, media was replaced every 2 days and glucose measurement was measured by glucose monitor(Roche). Glucose concentration did not drop below 4 mM in the normal glucose samples or below 25 mM in the high glucose samples. All experiments were repeated three times.

RNA extraction and quantitative real-time PCR

Cells were harvested for total RNA using Trizol according to manufacturer's protocol(Invitrogen, USA). Concentrations of total RNA were measured by spectrophotometry(260 nm/280 nm, Pharmacia 'DNA Calculator'). First-strand cDNA was synthesized from the total RNA(2 μg) using random hexamer primers and RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA). Quantitative real time PCR was run on MJ research OPTICON™ using SYBR Premix Ex Taq™ (Takara, Japan). Primers pairs for ABCA1[14], ABCG1 and GAPDH are listed in ***Tab 1***. Real time PCR thermocycling parameters were 95℃ for 10 sec, and 40 cycles of 95℃ for 5 sec, 54℃ for 20 sec and 72℃ for 12 sec. Gene levels were determined by the double delta method($2^{-\Delta\Delta Ct}$) with $\Delta Ct = Ct_{(\text{specific transcript})} - Ct_{(\text{GAPDH transcript})}$ and $\Delta\Delta Ct = \Delta Ct_{(\text{control})} - \Delta Ct_{(\text{treatment})}$. Real time PCR products were subsequently separated by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide.

Tab 1 **Quantitative real-time PCR primers used in this study**

Name		Sequence	Products(bp)	GenBank
ABCA1[14]	Forward	5-GCACTGAGGAAGATGCTGAAA-3	205 bp	NM005502
	Reverse	5-AGTTCCTGGAAGGTCTTGTTCAC-3		
ABCG1	Forward	5-TGCCAGGAAACAGGAAGATTAG-3	138 bp	NM207630
	Reverse	5-CACGAGACACCCACAAACC-3		
GAPDH	Forward	5-TCATCCCTGCCTCTACTG-3	175 bp	NM_002046
	Reverse	5-TGCTTCACCACCTTCTTG-3		

Western blot

RIPA buffer was added to VSMCs to generate whole cell lysate. Protein extracts(50 μg) were separated by 7% (for ABCA1) and 10%(for ABCG1) SDS-PAGE gel, and then transferred to nitrocellulose membrane using a Bio-Rad transfer blotting system. Non-specific binding was blocked with 5% skim milk for 2 h at room temperature. Blots were incubated overnight with rabbit polyclonal anti-ABCG1(1:200, SantaCruz, USA), rabbit polyclonal anti-ABCA1(1:2000,Novus Biologicals, USA) or β-actin antibody(1:5000). After washing with Tris-buffered saline+0.1% Tween-20, membranes were incubated for 1h at room temperature with a horseradish peroxidase conjugated anti-rabbit secondary antibody (1:5000). Antigen detection was performed with a chemiluminescence detection system (Syngene, England) and semiquantitative analysis of band was performed with Quantity One 4.3.1 software.

Statistical analysis

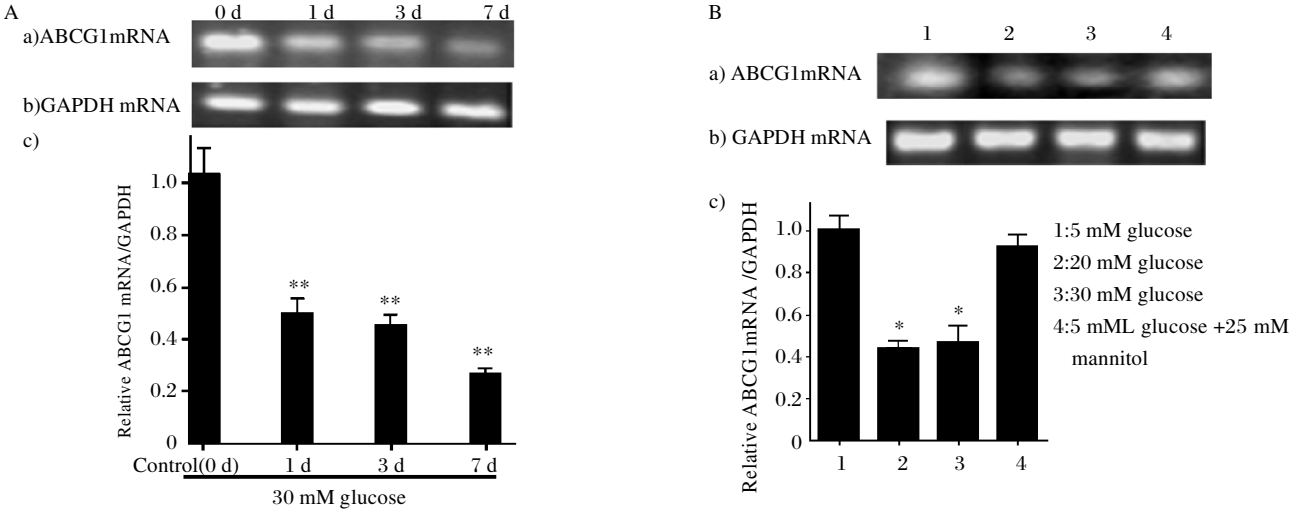
Statistical analysis was carried out by SPSS13.0. Data were graphically represented as mean ± SD; Differences between groups were examined using one-way ANOVA followed by Student's *t* test. *P* value < 0.05 was considered statistically significant.

RESULTS

Effect of D-glucose on VSMC ABC transporters mRNA expression

Real time PCR and PCR products electrophoresis showed that VSMC expressed not only high levels of ABCA1 mRNA but also ABCG1 mRNA. When VSMCs were cultured in 30mM glucose, ABCG1 mRNA expression was reduced by 50% at the first day

and further decreased by 73% at day 7 compared to controls(***Fig 1A***). When time was fixed at day 7, high glucose decreased ABCG1 mRNA expression, respectively reaching 43% and 46% of controls at 20 mM glucose and 30 mM glucose, whereas mannitol as an osmolality control did not change the ABCG1 mRNA expressions (***Fig 1B***).



Cells were cultured in 30 mM glucose for 0-7 days(A) or cultured in 5 mM, 20 mM, 30 mM glucose for 7 days, mannitol(5 mM glucose +25 mM mannitol) as an osmolality control(B). ABCG1 mRNA expression was determined by real time PCR as described in Methods. a) and b) present real time PCR products electrophoresis on a 1.5% agarose gel; c) real time PCR results analyzed by $2^{-\Delta\Delta Ct}$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. controls.

Fig 1 ABCG1 mRNA expression was decreased by high glucose in cultured VSMCs

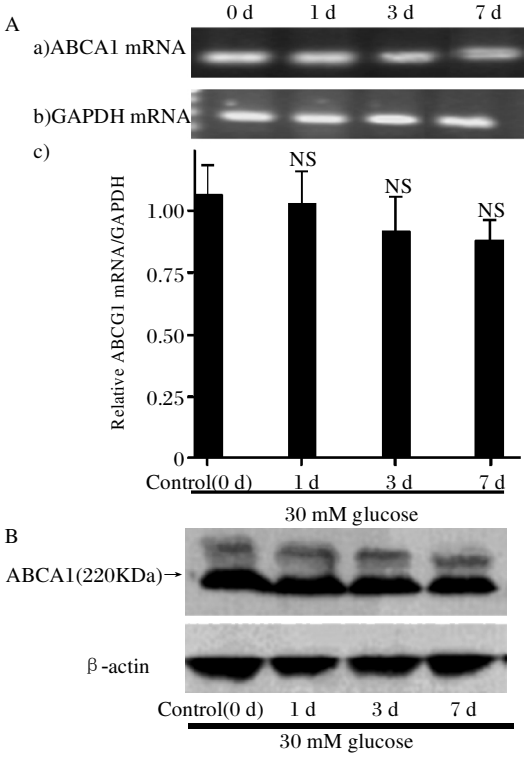
However, ABCA1 mRNA expression in VSMCs was not statistically influenced by high glucose, although a slight reduced trend with time course could be found (***Fig 2A***).

Effect of D-glucose on VSMC ABC transporters protein expression

We then examined whether ABCG1 protein expression decreased following the observed suppression of ABCG1 mRNA expression. Treatment of human VSMCs with 30 mM D-glucose reduced ABCG1 protein expression. This effect was observed at day 1, 3, 7, respectively 10%, 19%, 33% decrease compared with controls(***Fig 3A***). When VSMCs were cultured for 7 days in normal glucose and high glucose(20 and 30 mM D-glucose), ABCG1 protein expression was obviously decreased by high glucose. However, no significant reduction in ABCG1 protein expression was observed in mannitol-treated VSMCs(***Fig 3B***). ABCA1 protein expression showed no significant differences between each group of VSMCs(***Fig 2B***).

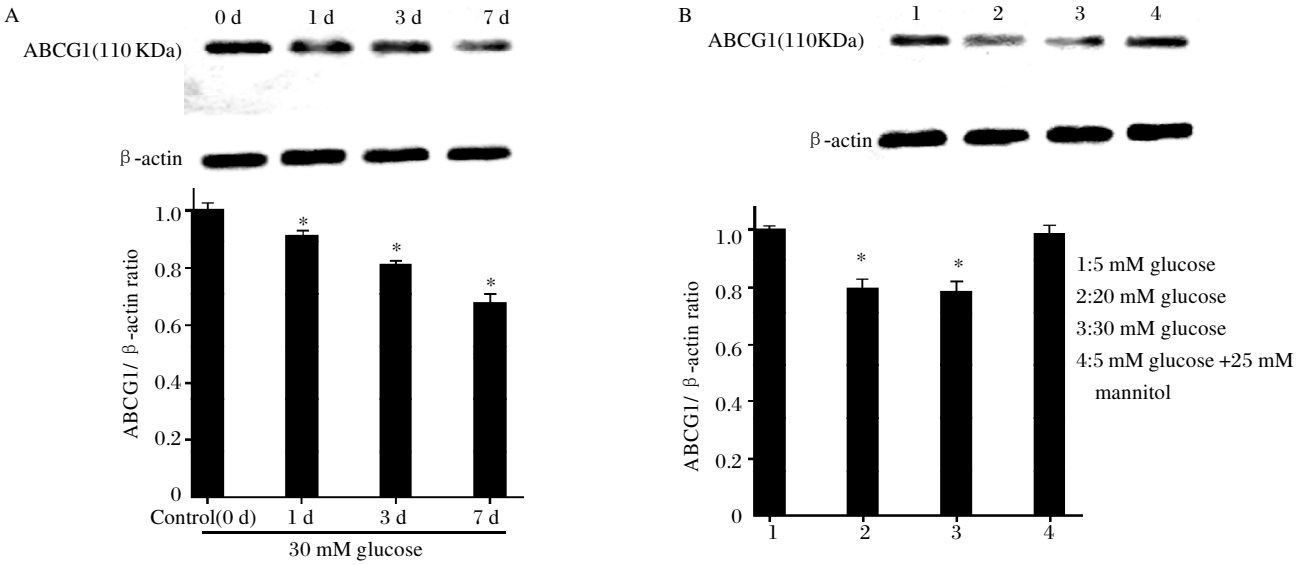
Role of oxidative stress in high glucose-mediated down-regulation of VSMC ABCG1

Studies have shown that diabetes and high glucose induce oxidative stress[3, 4]. To identify whether oxidative stress involved in the inhibitory effect of high glucose on ABCG1 gene expression, human VSMCs were pretreated for 1 hour with antioxidant NAC, then exposed



Cells were cultured in 30 mM glucose for 0-7 days, and ABCA1 mRNA expression was determined by real time PCR(A) and its protein expression was determined by Western blotting(B) as described in Methods. a) and b) present real time PCR products electrophoresis on a 1.5% agarose gel; c) real time PCR results analyzed by $2^{-\Delta\Delta Ct}$. NS=no significance

Fig 2 Effect of high glucose on ABCA1 mRNA(A) and protein(B) expression in cultured VSMCs



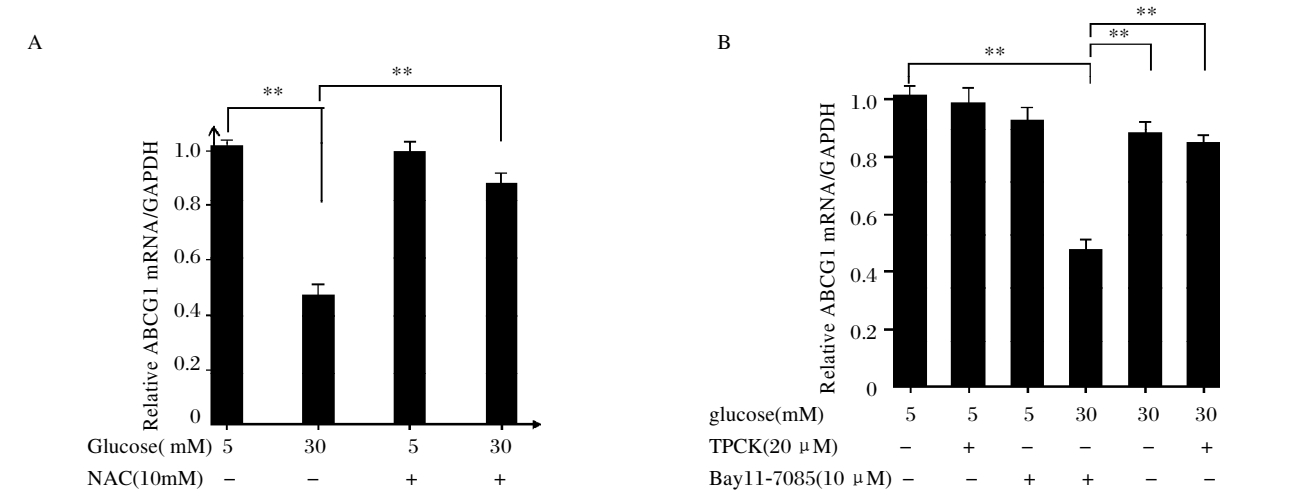
Cells were cultured in 30 mM glucose for 0-7 days(A) or cultured in 5 mM, 20 mM, 30 mM glucose for 7days, mannitol(5 mM glucose +25 mM mannitol) as an osmolality control(B). $^{*}P < 0.05$ vs. controls.

Fig 3 ABCG1 protein expression was decreased by high glucose in cultured VSMCs

to 5 mM and 30mM D-glucose. As shown in ***Fig 4A***, NAC almost totally abolished downregulation of ABCG1 mRNA expression in 30 mM D-glucose treated VSMCs. However, NAC had no effect on ABCG1 mRNA expression in VSMCs cultured in 5 mM glucose.

Role of NF-κB pathway in high glucose-mediated down-regulation of VSMC ABCG1

It has demonstrated that transcript factor NF-κB is activated by high glucose and diabetes[3,4]. So we determined the role of NF-κB in the regulation of ABCG1 gene expression. As shown in ***Fig 4B***, preincubation of VSMCs with two kinds of NF-κB inhibitors, Bay11-7085 and TPCK, almost totally prevented decrease in ABCG1 mRNA expression in 30 mM glucose-treated VSMCs. While in VSMCs incubated with 5mM glucose, no influence of NF-κB inhibitors on ABCG1 expression was observed.



ABCG1 mRNA expression was determined by quantative real time PCR as described in Methods. $^{**}P < 0.01$.

Fig 4 NAC(A) and NF-κB inhibitors(B) reverse the high glucose-mediated downregulation of ABCG1 mRNA expression in VSMCs

DISSCUSSION

VSMCs are known as one of the major components in atherosclerotic lesion and the origin of foam cells[15,16]. At present, much is known about the roles ABC transporters play in macrophage-derived foam cells[5-8], but little is known about the regulation of these proteins in VSMCs. We here reported that high glucose decreased expression of ABC transporter G1 in cultured VSMCs. Furthermore, glucose-induced downregulation of ABCG1 gene expression was reversed by NF-κB inhibitors as well as antioxidant, suggesting the regula-

tion of ABCG1 expression by oxidative stress and NF-κB pathway.

ABCG1 is a key regulator involved in cholesterol efflux to HDL and tissue lipid homeostasis[7, 9]. Recently, Kennedy *et al*[8] reported that targeted disruption of ABCG1 in mice resulted in massive accumulation of lipids in multiple tissues. In contrast, overexpression of ABCG1 protected murine tissues from dietary fat-induced lipid accumulation. They also found that ABCG1 was present in not only macrophages, but also lymphocytes, endothelial cells, hepatocytes, Kupffer cells, and epithelial cells. Here, our work extended this observation to VSMCs and showed that VSMCs also expressed ABCG1. Furthermore, ABCG1 expression in cultured VSMCs was repressed by elevated glucose. The result was consistent with what have observed in diabetic mouse peritoneal macrophages[11]. Thus, we hypothesized that downregulation of ABCG1 by high glucose could impact lipid trafficking in VSMCs, thereby contributing to VSMC-derived foam cell formation. However, further study on function of ABCG1 in VSMCs is in need.

The regulation of ABC transporters gene expression is redox sensitive[17, 18]. There are considerable evidences that diabetes and high glucose induce oxidative stress[3, 4]. Therefore, it is postulated that increased oxidative stress may represent key intermediate in the regulation of ABCG1 gene expression. In lines with this hypothesis, we found that antioxidant NAC, a scavenger of reactive oxidative species(ROS) abolished high glucose-induced suppression of ABCG1 mRNA expression in VSMCs. No effect of NAC on ABCG1 expression was shown in normal glucose, possibly due to low levels of oxidative stress under such a condition. It is suggested that increased oxidative stress as signaling event is involved in the regulation of ABCG1 gene in high glucose-treated VSMCs.

ABCA1 and ABCG1 share similar transcriptional control mechanism, including nuclear factor liver X receptors(LXR), peroxisome proliferator-activated receptors[6, 19]. However, here downregulation of ABCG1 gene by high glucose was not accompanied by decrease in ABCA1, identifying different regulation mechanism of these two transporters. It was reported that ABCG1 gene promoter had binding sites for NF-κB[20], and that binding of activated NF-κB to the promoter region of ABCG1 mediated ABCG1 transcriptional repression[21]. Therefore, it was speculated that blocking of activated NF-κB binding to ABCG1 promoter could induce ABCG1 transcriptional expression. Present results using NF-κB inhibitors supported our hypothesis, suggesting possible role of NF-κB activation in the regulation of ABCG1 gene expression. However, further study proposed to explore the relationship between NF-κB activity and ABCG1 gene expression.

In summary, present study showed that high glucose repressed the expression of ABCG1 in cultured VSMC, which is the possible mechanism of VSMC foam cell transformation. Nevertheless, additional studies are needed to assess the functional significance of downregulation of ABCG1 expression in VSMC derived foam cell formation.

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Understanding Wnt/b-catenin signaling in development and cancer

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The Wnt/beta-catenin signaling pathway plays key roles in development and diseases. This pathway requires two distinct receptors-a Frizzled serpentine receptor and LDL receptor related protein 5 or 6(LRP5 or LRP6). Among several areas that we are interested in is the mechanism of Fz-LRP5/6 receptor complex formation, activation and regulation. We have shown that Wnt induces LRP6 phosphorylation at multiple PPPSPxS motif, and this phosphorylation is necessary and sufficient to trigger Wnt signaling. Interestingly, GSK3 and CK1 are the kinases responsible for LRP6 phosphorylation. This dual-kinase mechanism for LRP6 activation is a mirror image of the dual-kinase mechanism of beta-catenin degradation we previously investigated, implying an intricate regulation of this pivotal Wnt pathway. I¹11 discuss recent our progresses in characterization of the mechanism of LRP6 signaling, and the regulation of LRP6 by varieties of agonists and antagonists. Our studies may have implications to cancer pathogenesis and therapy.