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Hyperglycemia Accelerates ATP-Binding Cassette Transporter A1 Degradation Via an ERK-Dependent Pathway in Macrophages

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ABSTRACT

An elevation in blood glucose concentration leads to increased risk of developing diabetes-associated atherosclerotic cardiovascular disease due to an excessive accumulation of cholesterol in arterial macrophages. ATP-binding cassette transporter A1 (ABCA1) is an atheroprotective protein that mediates the export of cholesterol from macrophages. The present study aims to investigate the effect of hyperglycemia on the regulation of ABCA1 expression and to explore its underlying mechanisms of regulation in macrophages. Our results show that high glucose activates the extracellular signal-regulated kinases (ERK) signaling pathway via reactive oxygen species (ROS) production, which in turn down-regulates ABCA1 mRNA and protein expression. This down-regulation is mediated by accelerating ABCA1 mRNA and protein degradation in macrophages exposed to high concentrations of glucose. Our results provide evidence for the first time that hyperglycemia inhibits ABCA1 expression by ERK-modulated ABCA1 mRNA and protein stability. Overall, these results provide a mechanism for hyperglycemia-induced reduction in ABCA1 expression, which suggests a promising strategy for the treatment of diabetes-associated atherosclerosis. J. Cell. Biochem. 114: 1364–1373, 2013.

KEY WORDS: HYPERGLYCEMIA; ABCA1 STABILITY; CHOLESTEROL EFFLUX; ERK; ROS

Hyperglycemia accelerates atherosclerotic lesion progression [Giacco and Brownlee, 2010; Reusch and Wang, 2011]. Clinical studies indicate that lowering glucose in patients with diabetes has a benefit for atherosclerosis [Brown et al., 2010; Mazzone, 2010]. The effect of diabetes on atherosclerosis is often accompanied by hyperlipidemia as well as low levels of circulating high density lipoprotein cholesterol (HDL-C) [Krentz, 2003]. HDL is a major lipoprotein exerting a functional effect on reverse cholesterol transport (RCT). RCT is the process by which excess cholesterol is removed from peripheral cells [Rosenson et al., 2012]. The role of ATP-binding cassette transporter A1 (ABCA1) in RCT and in the control of apolipoprotein AI (apoAI)-mediated cholesterol efflux has been well established [Oram and Vaughan, 2006]. Defects in the *ABCA1* gene lead to very low levels of circulating HDL in both Tangier disease and familial HDL deficiency [Singaraja et al., 2003]. In addition to ABCA1, ATP-binding cassette transporter G1 (ABCG1) is found to be associated with the regulation of RCT [Oram and Vaughan, 2006]. It has been reported that cholesterol efflux is decreased in peritoneal macrophages of diabetic mice compared to control mice [Gantman et al., 2010]. In a human study, both ABCA1 expression and cholesterol efflux are found to be impaired in patients with type 2 diabetes [Patel et al., 2011]. Nevertheless, the role of hyperglycemia in the regulation of ABCA1 expression and its underlying molecular mechanisms still remain to be elucidated.

Abbreviations used: ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; apoAI, apolipoprotein AI; ERK, extracellular signal-regulated kinases; HDL-C, high density lipoprotein cholesterol; JNK, c-Jun N-terminal kinase; LXR, liver X receptor; miRNA, microRNA; NAC, *N*-acetyl-L-cysteine; RCT, reverse cholesterol transport; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; HE, hydroethidine; ROS, reactive oxygen species; RXR, retinoid X receptor.

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The expression of ABCA1 is highly regulated at both transcriptional and post-transcriptional levels [Oram and Heinecke, 2005; Soumian et al., 2005]. The *ABCA1* gene is transcriptionally activated by liver X receptor (LXR) and retinoid X receptor (RXR), which form obligate heterodimers that then bind to their corresponding response elements within the *ABCA1* gene promoter [Schmitz and Langmann, 2005; Zhao and Dahlman-Wright, 2010]. In addition, several studies have reported that microRNAs (miRNAs) [Marquart et al., 2010; Ramirez et al., 2011] and ABCA1 phosphorylation [Martinez et al., 2003; Yamauchi et al., 2003] also play roles in affecting ABCA1 mRNA and protein stability, which in turn modulate ABCA1 expression. Hence, the aim of this study was to investigate whether hyperglycemia modulates ABCA1 expression via transcriptional and/or post-transcriptional regulation.

The levels of reactive oxygen species (ROS) are elevated in patients with diabetes and in streptozotocin-induced diabetic mice [Shen, 2010]; this is due to the fact that hyperglycemia promotes mitochondrial ROS generation and dysfunction [Dey and Swaminathan, 2010]. Exposing cultured vascular cells to high glucose also increases ROS production [Inoguchi et al., 2000]. A previous study reported that ROS are involved in interleukin-1β (IL-1β)-induced down-regulation of ABCA1 expression [Chen et al., 2007], suggesting that hyperglycemia-induced ROS generation may probably decrease ABCA1 expression. Moreover, hyperglycemia and ROS in type 2 diabetes have been reported to activate mitogen-activated protein kinase (MAPK) signaling cascades, which consist of the extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK), and p38 [Evans et al., 2002]. Therefore, this study addresses the hypothesis that hyperglycemia down-regulates ABCA1 expression via ROS-activated MAPK signaling cascades. We found that high glucose accelerates ABCA1 mRNA and protein degradation by ROS-activated ERK signaling cascade, which in turn decreases ABCA1 expression in macrophages. Our results provide evidence that hyperglycemia can regulate ABCA1 expression via a post-transcriptional mechanism.

MATERIALS AND METHODS

MATERIALS

D-glucose, *N*-acetyl-L-cysteine (NAC), SB239023 (p38 inhibitor), and SP600125 (JNK inhibitor) were obtained from Sigma–Aldrich (St. Louis, MO). U0126 (ERK inhibitor) was purchased from Calbiochem (San Diego, CA). Anti-ABCA1 and α-tubulin antibodies were obtained from Abcam (Cambridge, UK). Antibodies against phospho-p38, phospho-ERK1/2, phospho-JNK1/2, total p38, total ERK1/2, and total JNK1/2 were purchased from Cell Signaling Technology (Beverly, MA).

CELL CULTURE

Bone marrow-derived macrophages (BMDM) from 6- to 12-week-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were isolated and cultured as previously described [Chang et al., 2012]. The animal experiments were approved by the Animal Care and Utilization committee of National Yang-Ming University and followed by the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health). BMDM were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories GmbH, Linz, Austria), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml macrophage colony-stimulating factor (M-CSF; R&D Systems, Minneapolis, MN) at 37°C in 5% CO_2. Murine macrophage RAW264.7 cells were originally obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FBS at 37°C in 5% CO_2. All cell experiments were performed in 6-well plates containing different concentrations of glucose.

QUANTITATIVE REAL-TIME PCR AND REVERSE TRANSCRIPTION-PCR (RT-PCR)

Total cellular RNA was extracted using the TRI Reagent (Sigma–Aldrich) following the manufacturer's instructions. A total of 2 μg RNA was reverse-transcribed using moloney murine leukemia virus reverse transcriptase (MMLV; Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Finnzymes, Espoo, Finland) on a Roche LightCycler system (Roche Diagnostics, Mannheim, Germany). The primer sequences were as follows: ABCA1 (forward 5′-GGTTTGGAGATGGTTATA-CAATAGTTGT-3′ and reverse 5′-CCCGGAAACGCAAGTCC-3′), ABCG1 (forward 5′-AAGGCCTACTACCTGGCAAAGA-3′ and reverse 5′-GCAGTAGGCCACAGGGAACA-3′), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, forward 5′-GTATGACTCCACT-CACGGCAAA-3′ and reverse 5′-GGTCTCGCTCCTGGAAGATG-3′). Conventional PCR was performed using EmeraldAmp GT PCR Master Mix (Takara, Ohtsu, Japan). The following primers were used for the PCR amplifications: ABCA1 (forward 5′-CAATGCCCCTCTT-CATGACT-3′ and reverse 5′-TGCAGTGGTGAGATTGAAGC-3′) and actin (forward 5′-CCAGAGCAAGAGAGGTAT-3′ and reverse 5′-ATAGAGGTCTTTACGGATGT-3′). The PCR products were separated on 2% agarose gels, and visualized by ethidium bromide staining. The levels of *ABCA1* and *ABCG1* gene expression were normalized using actin or GAPDH as the internal control.

WESTERN BLOTTING

Cells were lysed using lysis buffer [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM dithiothreitol, complete protease inhibitor cocktail (Roche Diagnostics) and phosphatase inhibitor cocktail I and II (Sigma–Aldrich)]. The cell lysates were collected after centrifugation. The protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA; Sigma–Aldrich) as the standard. Equal amounts of protein were separated by SDS–PAGE and transferred to nitrocellulose membranes (Pall, Glen Cove, NY). The immunoblots were probed with the specific antibody overnight at 4°C and then incubated with goat anti-rabbit or mouse IgG horseradish peroxidase conjugated antibodies (Sigma–Aldrich). The protein bands were detected using the enhanced chemiluminescence method (ECL, PerkinElmer, Boston, MA). The intensity of the protein bands was quantified using Image Quant software (Molecular Dynamics, Sunnyvale, CA).

CHOLESTEROL EFFLUX

RAW264.7 macrophages were incubated with DMEM containing 0.5 μCi/ml [1,2-^{3}H]-cholesterol (PerkinElmer) and 0.2% BSA for 24 h at 37°C. Cells were washed and incubated with medium containing different concentrations of glucose for another 48 h. To perform the apoAI-mediated cholesterol efflux, cells were incubated with 10 μg/ml lipid-free human apoAI (Sigma–Aldrich) for 24 h. The medium was collected and cells were lysed in 0.1 N NaOH. The radioactivities of the medium and the cell lysate were measured by liquid scintillation counting. Cholesterol efflux was expressed as the percentage of counts in the media relative to counts in the media and cell lysate. ApoAI-specific cholesterol efflux was obtained by subtracting the nonspecific efflux that occurred in apoAI-free medium.

DETECTION OF INTRACELLULAR ROS PRODUCTION

The intracellular levels of ROS were determined using a fluorometric method. After glucose treatment, cells were washed and loaded with 20 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma–Aldrich) or 10 μM hydroethidine (HE, Sigma–Aldrich) for 30 min at 37°C. Fluorescent intensity was determined using a microplate fluorometer (Infinite 200, Tecan, Salzburg, Austria) with excitation wavelength at 485 nm and emission wavelength at 538 nm for DCFH-DA measurement or with excitation wavelength at 518 nm and emission wavelength at 605 nm for HE assay. The intracellular ROS levels were expressed as fold induction relative to the values obtained from the 5 mM glucose group.

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAYS

Different lengths of the mouse ABCA1 promoter sequences were amplified by PCR and cloned into the pGL3-basic luciferase vector (Promega, Madison, WI). RAW264.7 macrophages were transiently co-transfected with 1 μg of each of the various ABCA1 promoter-driven luciferase reporter plasmids and 0.5 μg of the pCMV-β-galactosidase expression plasmid using the Lipofectamine LTX and PLUS reagents (Invitrogen) according to the manufacturer's protocol. After glucose treatment for 48 h, cells were lysed and luciferase activity was determined using a VICTOR^2 Multilabel Reader (PerkinElmer). Luciferase activity was normalized to β-galactosidase activity and expressed as fold induction relative to the values obtained from the 5 mM glucose-treated pGL3-basic vector group.

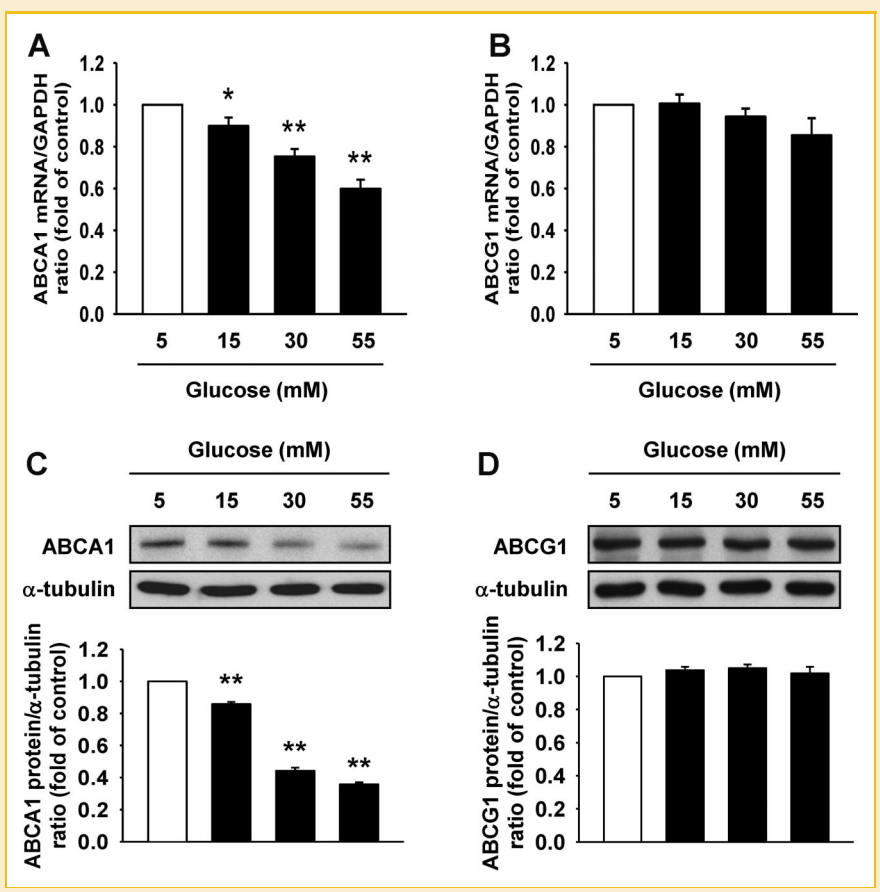

Fig. 1. Effects of high glucose on ABCA1 and ABCG1 expression in BMDM. BMDM were treated with different concentrations of glucose as indicated for 48 h. The mRNA levels of ABCA1 (A) and ABCG1 (B) were analyzed by quantitative real-time PCR and the protein levels of ABCA1 (C) and ABCG1 (D) were analyzed by Western blot. The normalized levels of mRNA or protein from cells with 5 mM glucose treatment are given the value of 1. Results represent mean ± SEM of at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ versus 5 mM glucose group.

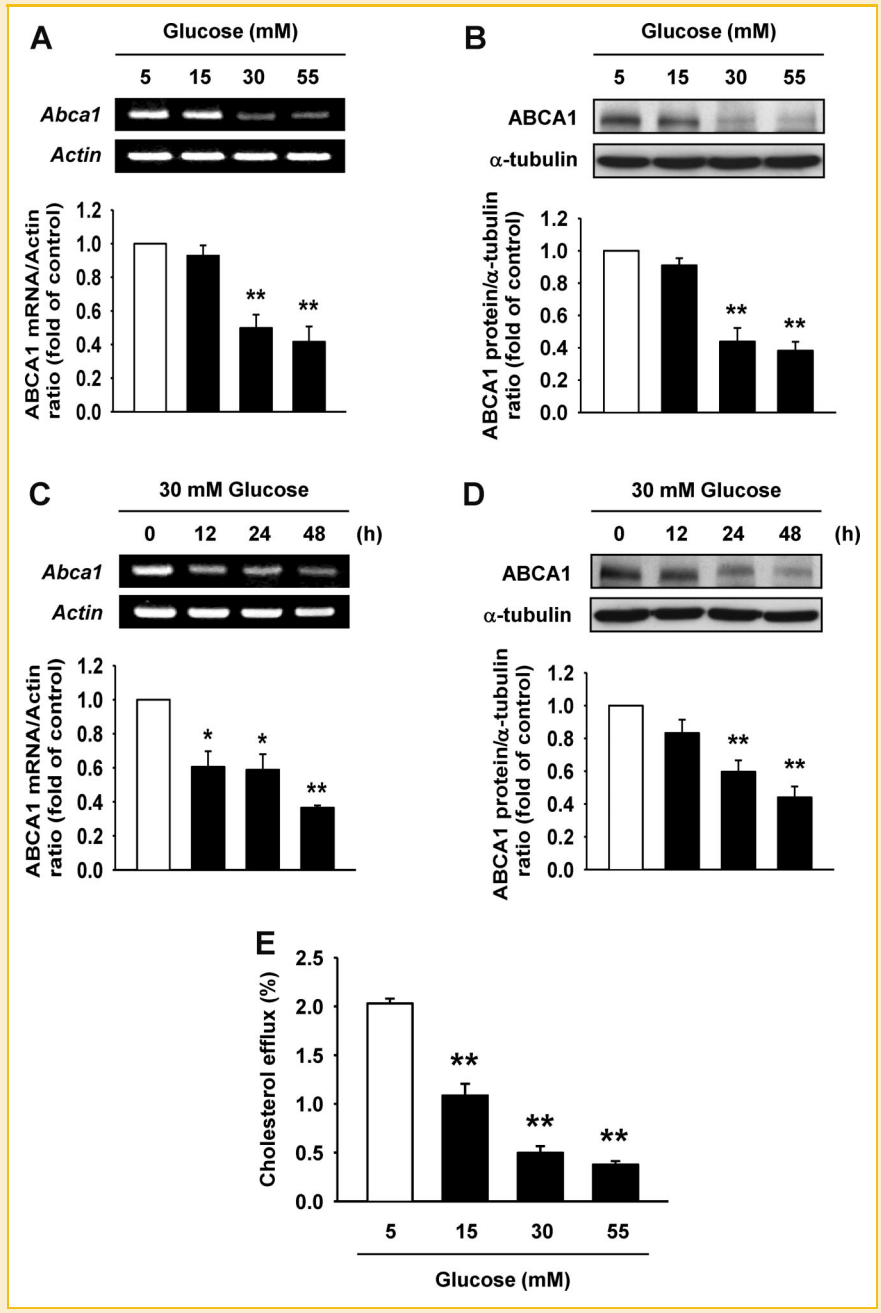

Fig. 2. Effects of high glucose on ABCA1 expression and cholesterol efflux in RAW264.7 macrophages. Cells were treated with different concentrations of glucose as indicated for 48 h. The levels of ABCA1 mRNA (A) and protein (B) were analyzed by RT-PCR and Western blot, respectively. The time-dependent effect of glucose on the levels of ABCA1 mRNA (C) and protein (D) were also analyzed in cells treated with 30 mM glucose. The normalized levels of mRNA or protein from cells with 5 mM glucose treatment are given the value of 1. E: ApoAI-mediated cholesterol efflux from RAW264.7 macrophages was measured as described in Materials and Methods Section. Results represent mean ± SEM of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus 5 mM glucose group.

DETERMINATION OF ABCA1 mRNA AND PROTEIN STABILITY

RAW264.7 macrophages were treated with 30 mM glucose in the presence or absence of 0.5 μM U0126 for 48 h before 5 μg/ml actinomycin D (Sigma–Aldrich) was added to stop new RNA synthesis. Total cellular RNA was extracted using the TRI Reagent at 0, 2, 4, and 6 h after the addition of actinomycin D. The mRNA levels of ABCA1 under the various conditions were determined by semi-quantitative RT-PCR as described above and the mRNA level of ABCA1 in 0 h group was set as 100%, with other time points expressed as relative percentage. To determine ABCA1 protein stability, 10 μg/ml cycloheximide (Sigma–Aldrich) was added to stop new protein synthesis after cells were treated with 30 mM glucose in the presence or absence of 0.5 μM U0126 for 48 h. Total cellular protein was harvested at 0, 2, 4, and 6 h after the addition of cycloheximide. The protein levels of ABCA1 under the various conditions were determined by Western blotting as previously

described and the protein level of ABCA1 in 0 h group was set as 100%, with other time points expressed as relative percentage.

STATISTICAL ANALYSIS

The results are presented as mean ± SEM of at least three independent experiments. Differences between control and treatment groups were assessed by Student's *t*-test. Differences were considered statistical significance at $P < 0.05$.

RESULTS

HIGH GLUCOSE REDUCES ABCA1 EXPRESSION AND CHOLESTEROL EFFLUX IN MACROPHAGES

To access the effect of high glucose on ABCA1 expression, BMDM were cultured in DMEM containing 15, 30, and 55 mM glucose for 48 h. As shown in Figure 1, high glucose significantly down-regulated the mRNA and protein levels of ABCA1 in a concentration-dependent manner compared to control cells cultured with 5 mM glucose. However, high glucose did not affect the mRNA and protein levels of ABCG1 in BMDM. We also observed that glucose dose-dependently down-regulated the mRNA and protein levels of ABCA1 in RAW264.7 macrophages (Fig. 2A,B). Moreover, the mRNA and protein levels of ABCA1 were decreased by 30 mM glucose in a time-dependent manner (Fig. 2C,D). We next examined the effect of high glucose on cholesterol efflux in macrophages. In line with the effect of high glucose on ABCA1 expression, treatment of cells with high glucose decreased cholesterol efflux from macrophages (Fig. 2E). These results indicate that high glucose-induced reduction in ABCA1 expression plays a pivotal role in the down-regulation of cholesterol efflux from macrophages.

HIGH GLUCOSE-INDUCED ROS PRODUCTION IMPAIRS ABCA1 EXPRESSION

To evaluate whether the high glucose-induced reduction in ABCA1 expression was correlated with ROS production, we examined intracellular ROS generation in RAW264.7 macrophages exposed to high concentrations of glucose by treating with DCFH-DA and HE dyes. The fluorescence of the oxidized DCFH-DA and HE was measured to evaluate the production of peroxides and superoxides, respectively. In Figure 3A, high glucose dose-dependently increased intracellular levels of peroxides by up to 1.7-fold. When cells were treated with NAC, a ROS scavenger, the levels of high glucose-induced peroxides were decreased (Fig. 3A). However, the intracellular levels of superoxides showed no change under high glucose challenge (Fig. 3B). We then investigated the effect of NAC on high glucose-induced suppression of ABCA1 expression. As

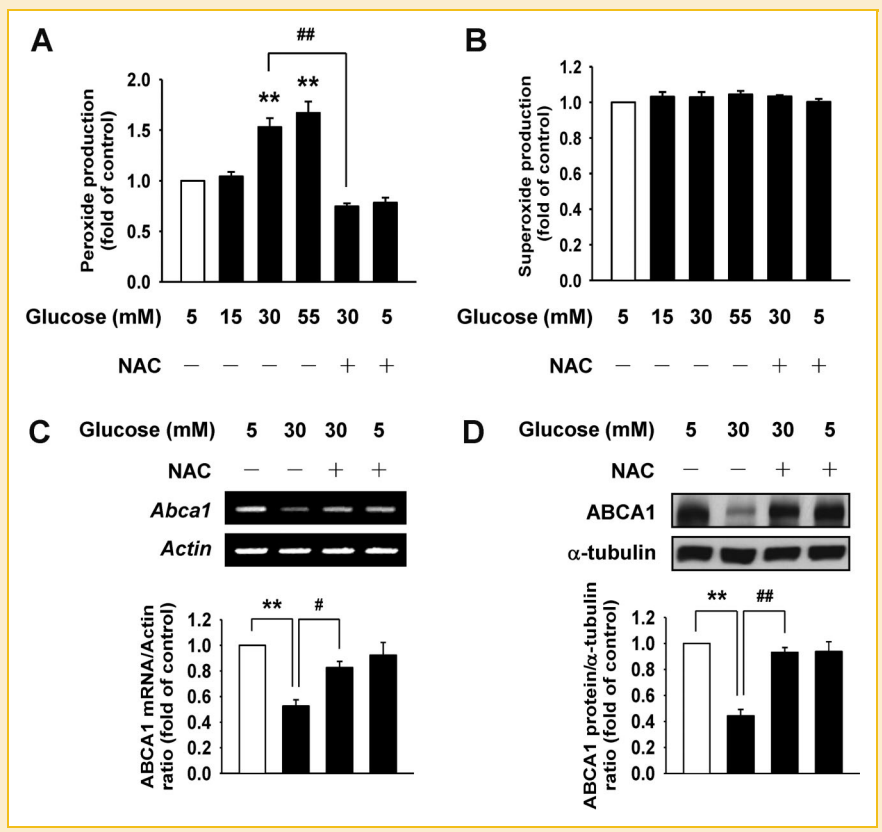

Fig. 3. Effects of high glucose-induced ROS production on ABCA1 expression. RAW264.7 macrophages were treated with glucose as indicated concentrations for 3 h and intracellular levels of peroxides (A) and superoxides (B) were measured as described in Materials and Methods Section. To confirm the effect of ROS on ABCA1 expression at mRNA (C) and protein (D) levels, cells were treated with 30 mM glucose in the presence or absence of 20 mM NAC, a ROS inhibitor. The histogram shows the relative fold compared with the 5 mM glucose group. The value from cells treated with 5 mM glucose is set as 1. Results are expressed as mean ± SEM of at least three independent experiments. $^{**}P < 0.01$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

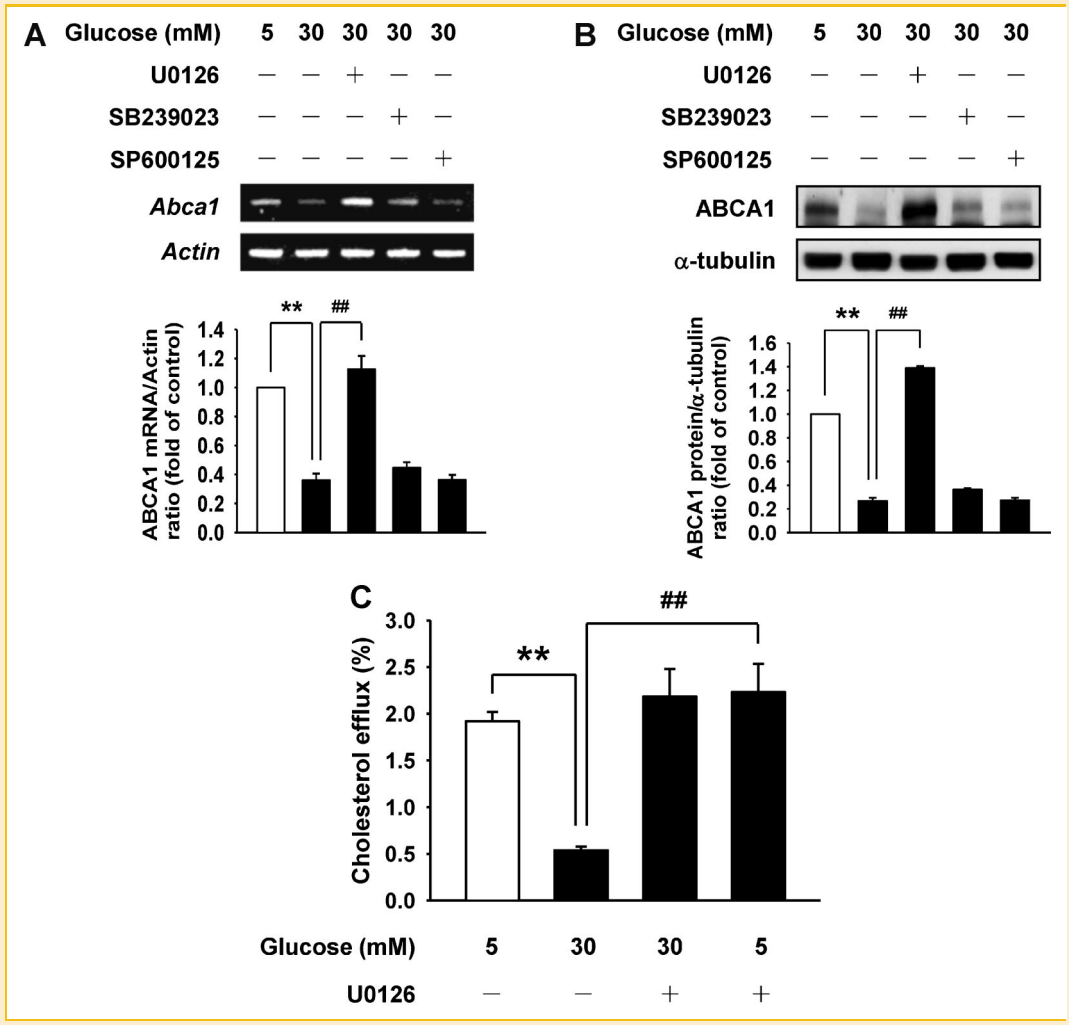

Fig. 4. Effects of MAPK inhibitors on high glucose-induced reduction in ABCA1 expression and cholesterol efflux. RAW264.7 macrophages were treated with glucose and inhibitors of ERK (U0126), p38 (SB239023), or JNK (SP600125) for 48 h. The levels of ABCA1 mRNA (A) and protein (B) were measured by RT-PCR and Western blot, respectively. The normalized levels of mRNA or protein from cells with 5 mM glucose treatment are set as 1. C: The effect of U0126 on high glucose-induced down-regulation of cholesterol efflux was also determined. Cells were treated with glucose and U0126 for 48 h and the cholesterol efflux was measured as described in Materials and Methods Section. Bars are mean ± SEM of at least three independent experiments. $^{**}P < 0.01$, $^{\#\#}P < 0.01$.

shown in Figure 3C,D, NAC reversed the suppressive effect of glucose on ABCA1 expression at both mRNA and protein levels in RAW264.7 macrophages, respectively. Taken together, these results indicate that ROS is essential for the high glucose-mediated reduction in ABCA1 expression.

HIGH GLUCOSE-STIMULATED ERK ACTIVATION REDUCES ABCA1 EXPRESSION AND CHOLESTEROL EFFLUX

To elucidate whether MAPK signaling pathway is involved in the reduction of ABCA1 expression by high glucose treatment, we examined the effects of ERK, p38 and JNK inhibitors on high glucose-suppressed ABCA1 expression. The ERK inhibitor, U0126, remarkably reversed the glucose-induced suppression of ABCA1 expression at mRNA and protein levels, whereas the glucose-modulated ABCA1 expression was not affected by the p38 and JNK inhibitors, SB239023 and SP600125, respectively (Fig. 4A,B). We also observed that U0126 significantly reversed the glucose-induced down-regulation of cholesterol efflux in macrophages (Fig. 4C). Furthermore, we assessed the effect of high glucose on MAPK phosphorylation. The phosphorylation level of ERK was significantly increased in RAW264.7 macrophages treated with 30 mM glucose for 9 h (Fig. 5A), while the high glucose did not increase the phosphorylation levels of p38 and JNK among the groups at various times compared to the 0 h group. These results indicate that ERK activation is involved in the glucose-induced reduction of ABCA1 expression and cholesterol efflux. Since the results in Figure 3A show that high concentrations of glucose increase intracellular peroxide production, we hypothesized that glucose-activated ERK phosphorylation may result from ROS production. As shown in Figure 5B, NAC significantly abolished the phosphorylation level of ERK induced by high glucose. These findings support the possibility that ROS is crucial for the high glucose-induced ERK activation, which in turn down-regulates ABCA1 expression and cholesterol efflux in RAW264.7 macrophages.

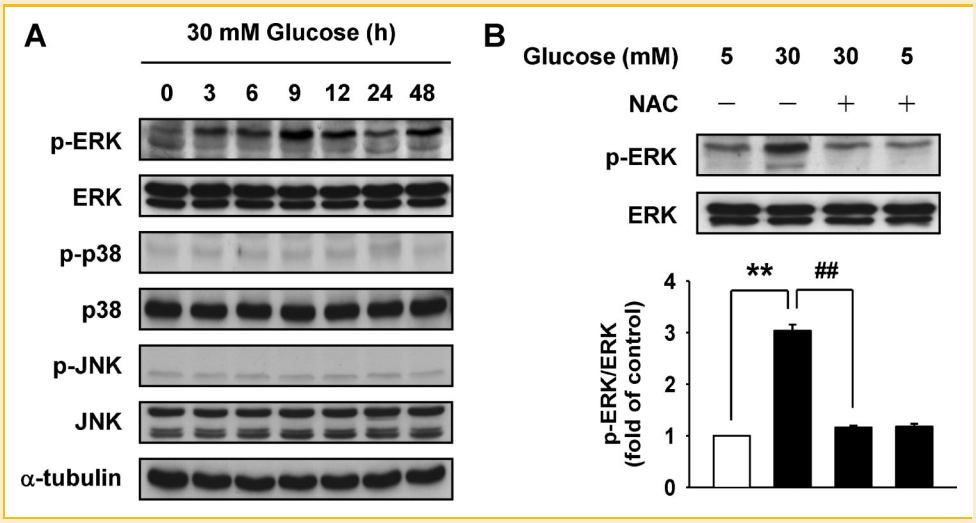

Fig. 5. Effects of high glucose on the activation of MAPK signaling pathways. A: RAW264.7 macrophages were treated with 30 mM glucose at the indicated times and the phosphorylation levels of ERK, p38, and JNK were measured by Western blot. B: To determine the effect of ROS on glucose-induced ERK phosphorylation, cells were treated with glucose in the presence or absence of NAC for 9 h. The histogram shows the relative fold compared with the 5 mM glucose group. The value from cells treated with 5 mM glucose is set as 1. Bars are mean ± SEM of at least three independent experiments. $^{**}P < 0.01$, $^{\#\#}P < 0.01$.

ERK IS CRITICAL FOR GLUCOSE-MEDIATED ACCELERATION OF ABCA1 DEGRADATION

To assess the effect of glucose on *ABCA1* gene promoter activity, 5′-serial deletion constructs, ABCA1-1100, ABCA1-700, ABCA1-400, and ABCA1-250, were co-transfected with a pCMV-β-galactosidase expression plasmid into RAW264.7 macrophages. Luciferase activity was normalized to β-galactosidase activity to correct for transfection efficiency. High glucose did not affect luciferase activity when compared to the respective controls (Fig. 6A), indicating that the reduction in ABCA1 expression caused by glucose is not due to transcriptional regulation. We then investigated whether high glucose alters the ABCA1 mRNA or protein stability. As shown in Figure 6B,C, cells treated with high glucose caused a significant increase in ABCA1 mRNA and protein degradation, whereas U0126 increased the ABCA1 mRNA and protein stability. Collectively, these results indicate that high glucose decreases the half-life of ABCA1 mRNA and protein via ERK activation.

DISCUSSION

Several studies have indicated that hyperglycemia, the major clinical hallmark of diabetes, increases the risk of atherosclerotic vascular disease [Nathan et al., 2003; Reusch and Wang, 2011]. Excess of cholesterol accumulation in arterial macrophages is the primary cause of atherosclerosis. It is believed that ABCA1-mediated cholesterol efflux from macrophages is the main process against atherosclerosis development [Moore and Tabas, 2011]. Recently, an animal study reveals that ABCA1 expression is decreased in arterial macrophages of diabetic swine compared to non-diabetic swine, which in turn augments atherosclerosis lesion development [Passarelli et al., 2005]. Moreover, hyperglycemia leads to a reduction in *ABCA1* gene expression in vascular smooth muscle cells (VSMCs) [Yu et al., 2010]. These studies suggest that hyperglycemia may contribute to down-regulation of ABCA1 expression in vascular cells. A recent study indicates that high glucose decreases ABCG1 but not ABCA1 expression in kidney mesangial cells [Song et al., 2012]. Another study reports that high glucose reduces both ABCA1 and ABCG1 expression in human macrophages [Mauerer et al., 2009]. It seems that the regulation of high glucose on ABCA1 and ABCG1 expression may differ in different cell types or animal species. However, our results clearly showed that ABCA1 expression, as well as cholesterol efflux, are decreased in BMDM and RAW264.7 macrophages exposed to high glucose concentrations, whereas there are no change in ABCG1 expression. In the present study, we also explored the molecular mechanisms involved in hyperglycemia-induced down-regulation of ABCA1 expression in macrophages.

ROS overproduction by mitochondria is a major cause of hyperglycemia-induced vascular damage [Giacco and Brownlee, 2010]. In our results, we observed that treatment of macrophages with glucose dose-dependently elicited an increase in peroxide production but not in superoxide production. This result is in agreement with studies showing that hyperglycemia induces hydroxyl radical production in streptozotocin-induced diabetic rats and rabbits [Ohkuwa et al., 1995; Winiarska et al., 2004; Frances et al., 2010]. Previous studies report that exposure of endothelial cells and mesangial cells with hyperglycemic concentrations of glucose increases the production of ROS [Inoguchi et al., 2000; Xia et al., 2008]. In addition, Chen et al. [2007] report that ABCA1 expression is down-regulated by IL-1β-induced ROS production. Accordingly, we hypothesized that hyperglycemia-induced ROS production may decrease ABCA1 expression in macrophages. This hypothesis was confirmed by the use of NAC, where it was found that NAC significantly reversed the inhibitory effect of high glucose concentrations on ABCA1 expression in macrophages. These

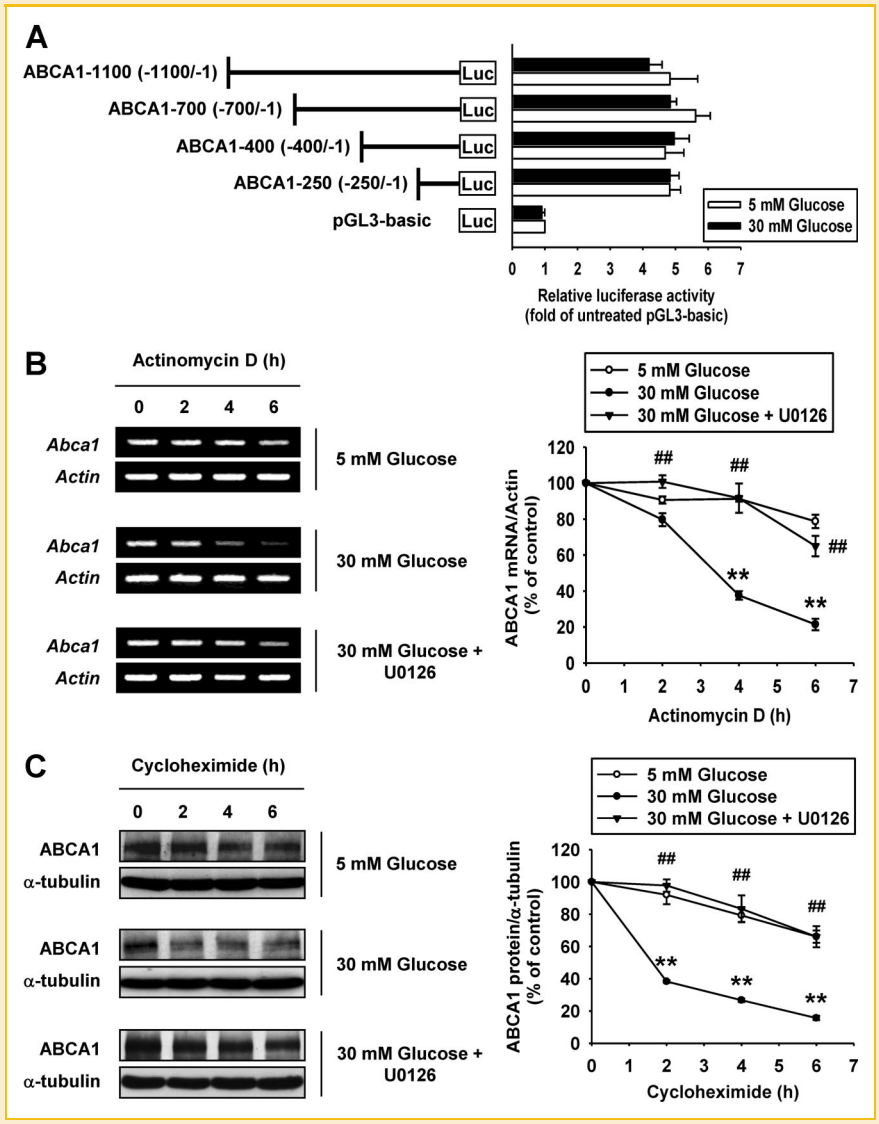

Fig. 6. Effects of high glucose on ABCA1 promoter activity and ABCA1 mRNA and protein stability. A: RAW264.7 macrophages were transiently transfected with 1 μg of ABCA1 promoter-driven luciferase reporter plasmid containing sequentially deleted 5′-flanking fragments as indicated, and then treated with or without 30 mM glucose for 48 h. A pCMV-β-galactosidase vector encoding β-galactosidase was co-transfected as an internal control. The luciferase activity was normalized to β-galactosidase activity and the relative induction fold was indicated as the relative value of untreated pGL3-basic vector (set as 1). B,C: Cells were incubated with glucose in the presence or absence of 0.5 μM U0126 for 48 h before the addition of 5 μg/ml actinomycin D or 10 μg/ml cycloheximide. Total cellular RNA and protein were extracted from the cells at indicated times, and the mRNA and protein levels of ABCA1 were determined by RT-PCR and Western blot, respectively. The mRNA or protein levels of ABCA1 at 0 h were defined as 100%, with other time points expressed as relative percentages. Values are mean ± SEM of at least three independent experiments. $^{**}P < 0.01$ versus 5 mM glucose group. $^{\#\#}P < 0.01$ versus 30 mM glucose group.

findings are similar to those of Xue et al. [2010] who showed that NAC reversed high glucose-suppressed expression of ABCG1 in VSMCs. Moreover, we also found that ERK was activated by high glucose-induced ROS. This activation is accompanied by reduced ABCA1 expression and cholesterol efflux. This observation is consistent with a study showing that activation of ERK accounts for VSMCs proliferation and is regulated by ROS [Ortmann et al., 2011]. In this study, we provide the first evidence that an activation of ERK via ROS production in macrophages plays an important role in the reduction of ABCA1 expression and cholesterol efflux that occurs in hyperglycemia.

A recent study has indicated that hyperglycemia suppresses ABCA1 expression via transcriptional regulation in VSMCs [Yu et al., 2010]. In contrast, we found that the promoter activity of ABCA1 did not change in the hyperglycemic groups compared to the control groups, suggesting that post-transcriptional regulation may be involved in the regulation of ABCA1 expression in high glucose-treated macrophages. Several lines of evidence report that microRNA-33 (miR-33) is a key regulator of cholesterol homeostasis including ABCA1 expression and that other miRNAs, for example miR-126, also show changes in diabetes subjects [Meng et al., 2012; Rayner et al., 2012]. miRNAs control post-transcriptional modula-

tion by altering mRNA stability and/or repressing mRNA translation [Rayner et al., 2012]. Furthermore, expression levels of several miRNAs, such as miR-21 and miR-221, have been reported to be induced via ERK signaling pathway [Ichimura et al., 2011]. In addition, phosphorylation of a PEST sequence in ABCA1 can decrease ABCA1 protein stability [Martinez et al., 2003]. We observed that high concentrations of glucose accelerated ABCA1 mRNA and protein degradation, decreased mRNA and protein stability, and these effects were reversed by ERK inhibition in RAW264.7 macrophages. This observation is in agreement with those of Zhou et al. [2010] who found that inhibition of ERK activation reduced the degradation of ABCA1 mRNA and protein in macrophages. Taken together, we clearly show that ERK activation enhances ABCA1 mRNA and protein turnover under hyperglycemia in macrophages. These results provide a possible mechanism whereby hyperglycemia down-regulates ABCA1 expression via post-transcriptional regulation.

In summary, this study shows a direct link between hyperglycemia and the down-regulation of ABCA1 expression. The underlying mechanisms whereby hyperglycemia induces suppression of ABCA1 expression are mainly via accelerated ABCA1 degradation, which is mediated by ROS-induced ERK activation. Therefore, glycemic control and ROS suppression would seem to be promising strategies for the prevention of atherosclerosis development in diabetes patients.

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