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Original Article

Exposure to High Glucose Concentration Decreases Cell Surface ABCA1 and HDL Biogenesis in Hepatocytes

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***Aim*: To study atherosclerosis risk in diabetes, we investigated ATP-binding cassette transporter A1 (ABCA1) expression and high-density lipoprotein (HDL) biogenesis in the liver and hepatocytes under hyperglycemic conditions.**

***Methods and Results*: In streptozotocin-induced diabetic mice, plasma HDL decreased while ABCA1 protein increased without changing its mRNA in the liver, only in the animals that responded to the treatment to show hypoinsulinemia and fasting hyperglycemia but not in the poor responders not showing those. To study the mechanism for this finding, hepatocytes were isolated from the control and diabetic mice, and they showed no difference in expression of ABCA1 protein, its mRNA, and HDL biogenesis in 1 g/l D-glucose but showed decreased HDL biogenesis in 4.5 g/l D-glucose although ABCA1 protein increased without change in its mRNA. Similar findings were confirmed in HepG2 cells with D-glucose but not with L-glucose. Thus, these cell models reproduced the *in vivo* findings in hyperglycemia. Labeling of cell surface protein revealed that surface ABCA1 decreased in high concentration of D-glucose in HepG2 cells despite the increase of cellular ABCA1 while not with L-glucose. Immunostaining of ABCA1 in HepG2 cells demonstrated the decrease of surface ABCA1 but increase of intracellular ABCA1 with high D-glucose. Clearance of ABCA1 was retarded both in primary hepatocytes and HepG2 cells exposed to high D-glucose but not to L-glucose, being consistent with the decrease of surface ABCA1.**

***Conclusions*: It is suggested that localization of ABCA1 to the cell surface is decreased in hepatocytes in hyperglycemic condition to cause decrease of HDL biogenesis.**

Key words: HDL, Cholesterol, ABCA1, Hyperglycemia, Atherosclerosis, Diabetes Mellitus

Introduction

Diabetes mellitus (DM) is a complex metabolic disorder primarily associated with dysfunction of insulin and accordingly hyperglycemia. In addition to specific complications such as diabetic retinopathy, neuropathy, and nephropathy, DM is known as a strong risk for atherosclerotic vascular diseases such as coronary heart disease and cerebral infarction. Noninsulin-dependent or type II diabetes associates with energy overtake and increase of body weight often accompanying insulin resistance, hyperinsulinemia, and overproduction of triglyceride-rich lipoprotein and accordingly elevation of low density lipoprotein (LDL), hypertriglyceridemia, hypertension, central obesity[1, 2], and decrease of high-density lipoprotein (HDL)[1, 3, 4]. In contrast, insulin-dependent or type I diabetes is caused primarily by deficiency of insulin secretion or of its activity to cause hyperglycemia, and therefore their metabolic disorder is substantially different from type II diabetes. Nevertheless, type I diabetic patients are also at high risk for atherosclerosis including coronary heart diseases[5] so that hyperglycemia itself may be recognized as an independent risk factor for cardio-

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vascular morbidity and mortality[6-8]. Those who develop atherosclerosis among type I patients were shown associated with low HDL cholesterol[9, 10].

Control of diabetes is in principle based on maintaining blood glucose level in the target range, and reduction for cardiovascular risk is achieved to some extent by this approach. However, it is not clear whether hyperglycemia is a real direct risk factor or just a surrogate marker for complex metabolic disorders in DM. Introduction of the inhibitors for renal sodium-dependent glucose co-transporter 2 (SGLT2)[11] provoked this argument as they target reduction of blood glucose level more directly than other approaches. The recent meta-analysis report indicated that this type of drug could reduce a cardiovascular risk[12]. We here intended to investigate direct involvement of hyperglycemia in atherogenic process focusing decrease of HDL.

HDL is generated by interaction of helical apolipoproteins such as apoA-I with cell membrane phospholipid and cholesterol in the presence of ATP-binding cassette transporter A1 (ABCA1)[13, 14]. This reaction is a major determinant of plasma HDL levels, and ABCA1 is a requisite and rate-controlling factor in this pathway. Mutations in ABCA1 impair apoA-I-mediated cellular lipids removal and cause genetic HDL deficiency Tangier disease[15-18], and its pharmacological inhibition by probucol reduces plasma HDL[19-21]. Genetic manipulations of ABCA1 expression in mice affect plasma HDL levels and atherogenesis[22, 23]. Liver-specific knockout of the ABCA1 reduced plasma HDL by 80% in mice[24] so that the liver seems to be a major organ to generate HDL by the ABCA1 pathway. The liver is also a major source of apoA-I, and HDL biogenesis takes place in hepatocytes as an autocrine reaction by interaction of the secreted apoA-I with their own ABCA1[25].

ABCA1 gene transcription in the liver is regulated sensing cell cholesterol level in a dual manner, positively by its liver X and retinoid X receptors[26] and negatively through sterol regulatory element-binding protein[27] or hepatic nuclear factor 4α[28], perhaps to control both production of HDL and flow of the recovered cholesterol from the body to conversion to bile acids. However, the reaction takes place on the cell surface so that cell surface ABCA1 is directly associated with HDL biogenesis[29], and its internalization is linked to calpain-mediated degradation that acts as a post-translational regulation system for ABCA1[30, 31]. Pharmacological inhibition of these reactions in fact increases surface ABCA1 and HDL biogenesis and reduces atherogenesis[32-34].

We here intended to investigate the influence of hyperglycemia on HDL metabolism, focusing on HDL biogenesis in hepatocyte as a main source for plasma HDL. We employed streptozotocin (STZ)-induced insulin-deficient mice as the compound selectively damages β-cells in the islets to cause primary hypoinsulinism and hyperglycemia with little other primary metabolic disorders. We here show that high glucose concentration reduces cell surface ABCA1 and HDL biogenesis in hepatocytes, the major site for HDL production.

Materials and Methods

Experimental Animals

C57BL/6 male mice at 10- to 14-month-old were injected with STZ, freshly dissolved in 0.1 M citrate buffer at pH 4.5 containing 0.15 M NaCl, at an intraperitoneal dose of 4 mg/50 g body weight to develop insulin-deficient diabetes[35, 36]. Plasma fasting (12 h) glucose was monitored for 0, 1, 2, and 3 weeks. The STZ-treated animals were divided into the responder group that showed hypoinsulinemia and severe fasting hyperglycemia (STZ-hyperglycemia [STZ-HG]) and the poor responder group that showed fasting normoinsulinemia and normoglycemia with moderately reduced insulin response to feeding (STZ-normoglycemia [STZ-NG]; **Table 1**). The experimental protocol was approved by the Animal Welfare Committee of the Nagoya City University Graduate School of Medical Sciences according to the institutional guidelines (approval no. H17-15).

Cell Culture

Mouse primary hepatocytes were harvested by the EDTA-collagenase two-step perfusion method as described previously[25, 37] at a concentration of 0.2×10^6 cells/ml in collagen-coated plate Dulbecco's modified Eagle medium (DMEM) containing 17% of fetal bovine serum (FBS) for 2 h in the presence of low (1 g/l) and high (4.5 g/l) D-glucose. Medium was then changed to DMEM containing 0.02% bovine serum albumin (BSA) and 1 or 4.5 g/l-glucose followed by incubation for 16 h in CO_2 incubator at 37℃ to examine ABCA1 expression and HDL biogenesis. Human hepatoma cell line, HepG2 cells, were cultured in 0.6×10^6 cells/ml in 10% FBS containing minimum essential medium (MEM) alpha modification (Sigma) containing D-glucose 1 g/l. Cells at 80%–90% confluence were exposed to additional 1 or 4.5 g/l D- or L-glucose in the medium containing 0.02% BSA for 16 h, and ABCA1 expression and HDL biogenesis were examined[25]. The cells consume D-glucose to reduce its concentration in the medium by approximately 1 g/l after this incubation.

Table 1. Characterization of diabetes induced by STZ in C57/BL/6 mice. Control represents mice without STZ treatment. STZ-hyperglycemia (STZ-HG) represents the STZ-treated mice that developed hyperglycemia with impaired response of insulin to feeding. STZ-normoglycemia (STZ-NG) indicates the mice treated with STZ but did not develop diabetes. BG: blood glucose. Data represent mean ± SD for n specified. Difference from control is indicated as $^{**}p<0.001$, $^{*}p<0.01$. "Fasting" indicates 12-hour fasting.

Week	0	1	2	3	
Control ($n=4$)					
Body Weight	36.1 ± 3.7	34.0 ± 3.8	33.3 ± 3.3	32.5 ± 3.6	(g)
Fasting BG	45.0 ± 3.8	38.3 ± 4.2	45.8 ± 4.2	49.6 ± 5.6	(mg/dl)
Fasting insulin	2.46 ± 0.05	3.09 ± 0.07	2.40 ± 0.03	----	(ng/ml)
Fed BG	----	----	92.9 ± 6.0	----	(mg/dl)
Fed insulin	----	----	88.9 ± 0.9	----	(ng/ml)
STZ-HG ($n=4$)					
Body Weight	34.9 ± 8.0	30.4 ± 6.2	28.5 ± 5.2	28.1 ± 4.9	(g)
Fasting BG	31.5 ± 1.6	138.7 ± 9.2	144.5 ± 10.2**	143.7 ± 13.3**	(mg/dl)
Fasting insulin	2.96 ± 0.05	2.19 ± 0.13	1.55 ± 0.17	----	(ng/ml)
Fed BG	----	----	473.8 ± 8.9**	----	(mg/dl)
Fed insulin	----	----	2.15 ± 0.13**	----	(ng/ml)
STZ-NG ($n=6$)					
Body Weight	35.1 ± 5.0	32.6 ± 3.1	32.4 ± 2.5	30.9 ± 2.4	(g)
Fasting BG	33.4 ± 3.8	46.0 ± 10.5	51.5 ± 10.0	47.3 ± 12.3	(mg/dl)
Fasting insulin	2.88 ± 0.01	2.04 ± 0.01	2.50 ± 0.04	----	(ng/ml)
Fed BG	----	----	98.7 ± 20.5	----	(mg/dl)
Fed insulin	----	----	28.0 ± 0.9*	----	(ng/ml)

Plasma Insulin and Glucose Assay

Mouse plasma insulin level was measured in fasting and fed conditions with enzyme-linked immunosorbent assay using an insulin kit (Morinaga, Japan) for 5-µl plasma/assay. Mouse plasma glucose level was analyzed using colorimetric enzymatic assay (Wako, Japan) for 2 µl plasma in both fed and fasting conditions.

High-Performance Liquid Chromatography Analysis of Mouse Plasma

Mice plasma, 5 µl, was analyzed in high-performance liquid chromatography (HPLC) using a Tricorn 5/200 GL column (207 × 5 mm, GE Healthcare Life Sciences) equilibrated with 0.05 M phosphate buffer at pH 7.4 containing 0.15 M NaCl (PBS) at a flow rate of 0.3 ml/min[38]. TC and PL were measured in the eluted fractions (100 µl each) with colorimetric enzymatic assay systems (Kyowa Medics, Japan).

Ultracentrifugation Analysis of the Culture Medium

Lipoprotein fractions in culture media were analyzed by ultracentrifugation[25]. Density of the medium was adjusted to 1.063 g/ml with NaBr and centrifuged at 49 k rpm for 16 h at 4℃ to collect the very low and low density lipoprotein fraction as a top fraction, and the remaining bottom part was adjusted to 1.21 g/ml and centrifuged similarly for 24 h to collect the floated HDL fraction. Lipid was extracted from each fraction with four times volume of chloroform: methanol (C/M = 2:1) overnight and determined for total cholesterol and choline-phospholipid using colorimetric enzymatic assay systems (Kyowa Medics and Wako, Japan, respectively).

Western Blotting Analysis of ABCA1

ABCA1 protein in hepatocytes was analyzed by Western blotting. Bulk cell membrane fraction was prepared according to the method previously described[25, 39]. The membrane fraction, 60 µg protein, was analyzed by Western blotting using a specific polyclonal antibody against a C-terminal peptide of human ABCA1 that cross reacts mouse ABCA1 as described previously[25, 39].

Labeling of Surface ABCA1

HepG2 cells were labeled with ^{125}I (1 µCi) using IODO-BEAD® iodination reagent (Piece). After washing the unbound ^{125}I with PBS, the bulk membrane fraction of the labeled cells was collected as described above. The membrane fraction, 500 µg, was mixed and rotated with control IgG-bound protein A-agarose for 1 h at 4℃. The supernatant was then allowed for specific binding with specific antibodies against human

ABCA1 or integrin β1 or antimouse scavenger receptor B1 (SRB1) that cross reacts with the human counterpart, CLA-1, bound with protein A-agarose by rotation for 2 h at 4℃. Pellet from this reaction was washed with cold lysis buffer and PBS and then dissolved in a solvent containing 9 M urea, 2% Triton X-100 and 1% dithiothreitol and analyzed in 6% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was dried and incubated with the imaging plate for 2 days, and the ^{125}I-labeled protein was detected by the BAS-1800II Fujifilm. Alternatively, cell surface protein was labeled with sulfosuccinimidyl 2-(biotinamido)-rthyl-1, 3-dithiopropionate (sulfo-SS-biotin; Pierce), 0.8 mM, for 1 h at 4℃ as previously reported[29]. After quenching the reaction, cells were washed and lysed, and the biotinylated membrane proteins were isolated by coprecipitation with streptavidin-agarose beads (Sigma) at 4℃ for 1 h. Proteins bound to the beads were eluted with the SDS sample buffer and analyzed in immunoblotting using a specific antibody against ABCA1 or integrin β1.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from cells using RNA extraction reagent (Isogen, Nippon Gene). Single-strand cDNA was synthesized by a SuperScript™ preamplification system (Invitrogen) from 5 mg of the total RNA. Polymerase chain reaction (PCR) was carried out for cDNA using primers (sense and antisense) of human ABCA1 (5′-GAA CTG GCT GTG TTC CAT GAT-3′ and 5′-GAT GAG CCA GAC TTC TGT TGC-3′), human apoA-I (5′-AGA GAC TGC GAG AAG GAG GTG-3′ and 5′-CAG ATC CTT GCT CAT CTC CTG-3′), mouse ABCA1 (5′-CTC AGA GGT GGC TCT GAT GAC-3′ and 5′-CCC ATA CAG CAA GAG CAG AAG-3′), mouse apoA-I (5′-ACG TAT GGC AGC AAG ATG AAC-3′ and 5′-AGA GCT CCA CAT CCT CTT TCC-3′), mouse β-actin (5′-ATG GTG GGA ATG GGT CAG AAG-3′ and 5′-CAC GCA GCT CAT TGT AGA AGG-3′) (synthesized by Hokkaido System Science, Japan). Quantification of mRNA for these primer products were accomplished using SYBR Green PCR master mix reagent in an AB." PRISM 7700 sequence detection system (Applied Biosystems, Japan).

Immunofluorescent Staining of Cells

HepG2 cells cultured in D- and L-glucose at 80% confluence were washed two times with cold PBS, fixed in 2% paraformaldehyde at 4℃ for 20 min and treated with 0.1% Tween-20 for 5 min × 2 at room temperature. After fixation, the cells were blocked with PBS containing 1% BSA and 0.05% Tween-20. After washing with PBS, the chamber slide cultures were incubated with rat antihuman ABCA1 (1:30, v/v) for 48 h, washed three times with PBS, and incubated for 30 min with goat anti-rat IgG-fluorescein isothiocyanate (1:100 SANTA CRUS) to visualize the protein with green emission colors. After washing three times with PBS, the chamber slides were enclosed with Dapi-Fluoromount-G (SouthernBiotech) for staining nuclei and examined in a KEYENCE BZ90000 immunofluorescence microscope (KEYENCE). BZ-II Measurement Module v1.01 BZ-H1M was used to obtain RGB photo intensity by green for ABCA1 and blue for nuclei.

Analysis of ABCA1 Clearance

Clearance rate of ABCA1 was evaluated as described previously[39]. HepG2 cells in 60-mm dishes were incubated in MEM with additional 0, 1, and 4.5 g/l D- and L-glucose. Cells were washed three times with PBS and incubated at 37℃ in MEM (alpha modification) containing 20 μg/ml cycloheximide (Wako Pure Chemicals) for 0, 0.5, 1, 1.5, and 2 h. ABCA1 in the cell membrane fraction was detected by Western blotting analysis.

Other Methods

Human apolipoprotein A-I was suspended in PBS in concentrations of 2, 5, 10, 15, and 20 μg/ml in the presence and absence of D- or L-glucose. Circular dichroism spectra were measured using Spectropolarimeter J-725 (JASCO, Japan) at 37℃. Protein content of each sample was determined with the bicinchoninic acid assay reagent (Pierce). Density of the visualized bands was semi-quantified by digital scanning in an EPSON GT-X700 and Adobe Photoshop software. Statistical significance of the differences between groups was evaluated using two-tailed Student's t test for unpaired comparisons or ANOVA for small sample sizes. $p < 0.05$ was considered significant. Values are expressed as mean ± SD.

Results

C57BL/6 mice were treated with STZ to induce insulin-deficient diabetic conditions. Four mice among the 10 treated developed diabetes as indicated by low insulin and high glucose levels in plasma (STZ-HG) while remaining 6 mice apparently remained with normal fasting plasma insulin and glucose levels except that their response of insulin to feeding was moderately impaired (STZ-NG), in comparison with the control mice (**Table 1**). Plasma HDL levels of these animals were monitored for both cholesterol and phos-

pholipid using the HPLC analyses (**Supplementary Fig. 1**) and shown reduced both in HDL cholesterol and phospholipid in those that developed diabetes by STZ but neither in the STZ-untreated control nor in the STZ-treated mice that did not develop diabetes (**Fig. 1A**). In spite of decrease of plasma HDL, the liver ABCA1, a major determinant of plasma HDL biogenesis, apparently increased in the diabetic mice in comparison with the control and the STZ-treated nondiabetic mice (**Fig. 1B**). Bip/GRP78 was decreased in the diabetic animals reflecting their insulin-deficient metabolic status, being consistent with the previous publication[40] so that integrin β1 was also used as an additional loading control (**Fig. 1B**). Since STZ-treated mice are not likely to be influenced by this dose in the organs and the cells other than β-cells, the effects of the condition are likely to be caused by hypoinsulinism or hyperglycemia. The mRNA level of ABCA1 in the liver was not different among these groups (**Fig. 1C**).

The primary hepatocytes were isolated from the animals and analyzed for expression of ABCA1 and HDL biogenesis in culture. ABCA1 protein level showed no difference between the cells isolated from diabetic and control mice in low glucose media (1 g/l) but increased by high D-glucose (4.5 g/l), regardless of the diabetic and normal (**Fig. 2A**). As the culture is not in an insulin-deficient condition, Bip/GRP78 level stayed stable by changing glucose concentration so much as integrin β1 and β-actin (data not shown). In spite of the increase of ABCA1, HDL biogenesis was decreased in both diabetic and control cells in high glucose culture medium (**Fig. 2B**). The findings were same even when the initial primary culture medium that contained high glucose by the original protocol was replaced with that of low glucose (data not shown). The mRNA for ABCA1 and apoA-I was both uninfluenced by change of glucose concentration in the medium (**Fig. 2C**). Thus, the findings in diabetic model mice were reproduced with their primary hepatocytes in culture, not by the source of the cells but by exposing the cells to high glucose concentration regardless of the cell sources.

HepG2 cells were analyzed in a similar manner for their ABCA1 expression and HDL biogenesis. Since HepG2 cells consume glucose to reduce its concentration by 1 g/l during the incubation period employed, the experiments were carried out in the presence of additional glucose in the medium. ABCA1 protein expression increased when additional concentration of D-glucose increased from 1 to 4.5 g/l in the culture medium but not by L-glucose (**Fig. 3A**). HDL biogenesis in contrast decreased by the increase of D-glucose concentration but remained same with the L-glucose increase (**Fig. 3B**). The mRNA of ABCA1 and apoA-I was both increased when 1 g/l D-glucose added but not in the other conditions (**Fig. 3C**). The results with HepG2 cells were thus consistent with those of primary hepatocytes with respect to the effects of high glucose on ABCA1 expression and HDL biogenesis, except for the increase of the messages at 1 g/l D-glucose added.

To understand the underlying mechanism for the decrease of HDL biogenesis by high glucose *in vivo* and *in vitro* despite the increase of overall cellular ABCA1, cell surface ABCA1 was estimated in HepG2 cells by labeling surface ABCA1 with biotin (**Fig. 4**) or ^{125}I (**Fig. 5**). The biotin-labeled ABCA1 decreased by increasing the additional D-glucose concentration from 1 to 4.5 g/l (**Fig. 4**). The ^{125}I-labeled surface ABCA1 also decreased by the increase of D-glucose in the medium (**Fig. 4A**). The ratio of surface to total cellular ABCA1 significantly decreased in high additional D-glucose condition. The surface ABCA1 level was therefore consistent with the changes of the HDL biogenesis shown in **Fig. 3B**. L-Glucose concentration much less influenced surface ABCA1.

Intracellular distribution of ABCA1 was examined by its immunostaining in HepG2 cells. Typical profiles are shown in **Fig. 6** to demonstrate that ABCA1 increased only when the cells were incubated under additional D-glucose at 4.5 g/l. The quantitated data are illustrated as a graph of green/blue as an indicator of ABCA1/cell. Intracellular increase of ABCA1 induced hyperglycemic condition was observed, predominantly in the perinuclear regions, being consistent with the results of surface ABCA1 labeling experiments described above.

Clearance rate of ABCA1 protein was analyzed (**Fig. 7**). It was previously shown that the secreted endogenous apoA-I interacts with cell surface ABCA1 in an autocrine manner to generate HDL in hepatocytes[25]. It was also demonstrated that apoA-I protects ABCA1 from the calpain-mediated degradation in the HDL biogenesis reaction[30, 41]. ABCA1 clearance was in fact slower in the primary hepatocytes than the rate in such cells as not secreting apolipoproteins such as fibroblasts (**Fig. 7A**). The rate of the ABCA1 decay was further slower in the hepatocytes in high glucose medium being consistent with its increase in this condition (**Fig. 4A**). The decay rate of ABCA1 in HepG2 cells was also retarded only in the high D-glucose medium among the control, low, and high D- and L-glucose media (**Fig. 7B**). The finding of retarded ABCA1 degradation was consistent with the observation that cell surface ABCA1 decreased when hepatocytes are exposed to high glucose environment.

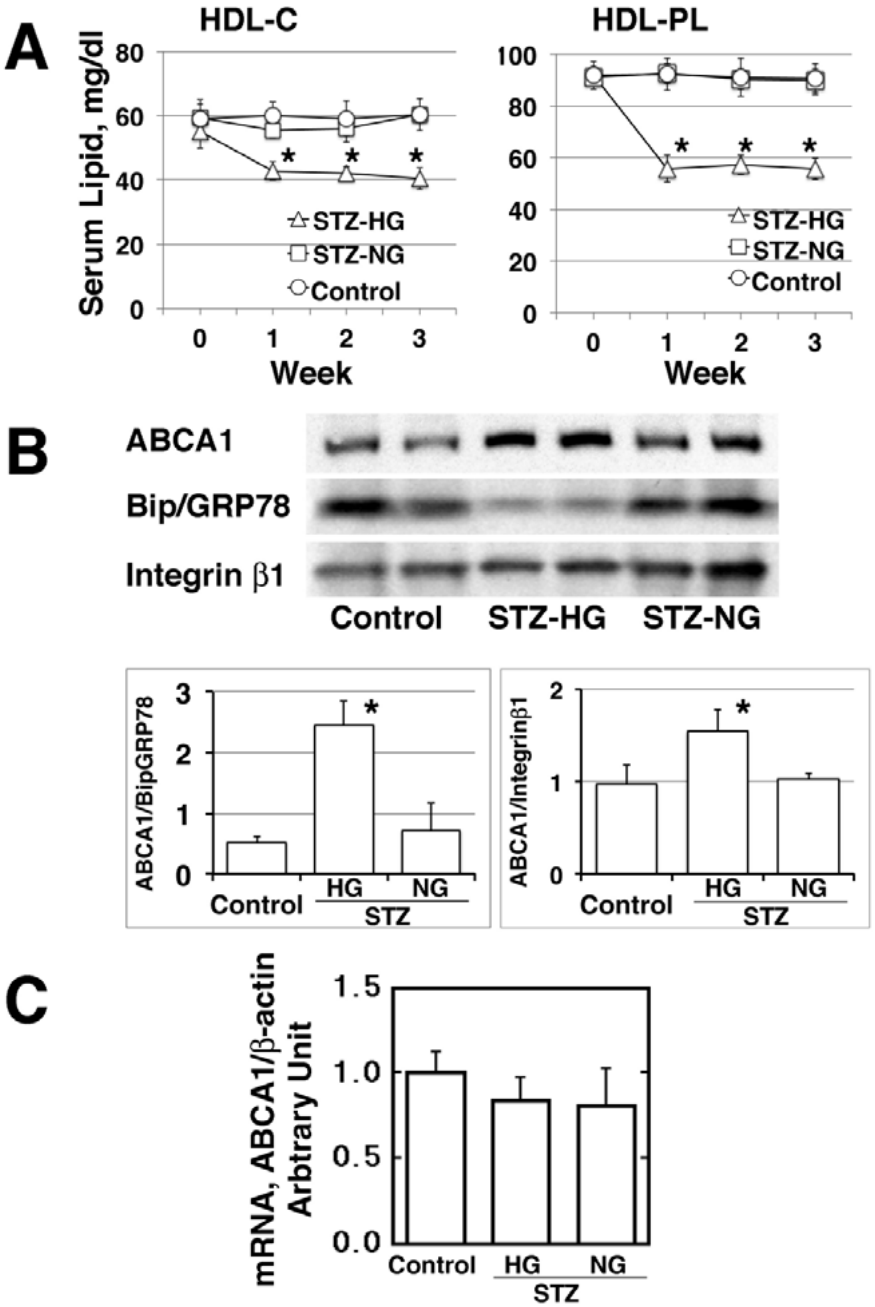

Fig. 1. Change in plasma HDL and hepatic ABCA1 expression in diabetic mice. BL57/6B mice were treated with STZ to induce diabetes, and plasma lipoproteins were analyzed by HPLC. A: Plasma HDL concentration in mice. HDL-C and HDL-PL indicate cholesterol and choline-phospholipid in HDL. Control indicates the mice without treatment ($n=4$), STZ-HG indicates fasting hyperglycemic mice induced by STZ ($n=6$), and STZ-NG indicates the normoglycemic mice even after the treatment with STZ. B: ABCA1 protein in the liver of the mice. ABCA1, Bip/GRP78, and integrin $\beta 1$ were analyzed for the liver homogenates by Western blotting. Control, STZ-HG, and STZ-NG are defined as above. Since Bip/GRP78 was shown decreased in the hyperglycemic mice, ABCA1 was standardized by integrin $\beta 1$ as well. C: Expression of mRNA of ABCA1 in the liver of the mice of control, diabetic, and nondiabetic after the STZ treatment analyzed by real-time RT-PCR. Asterisks indicate significant difference of DM from control and STZ by $p<0.05$.

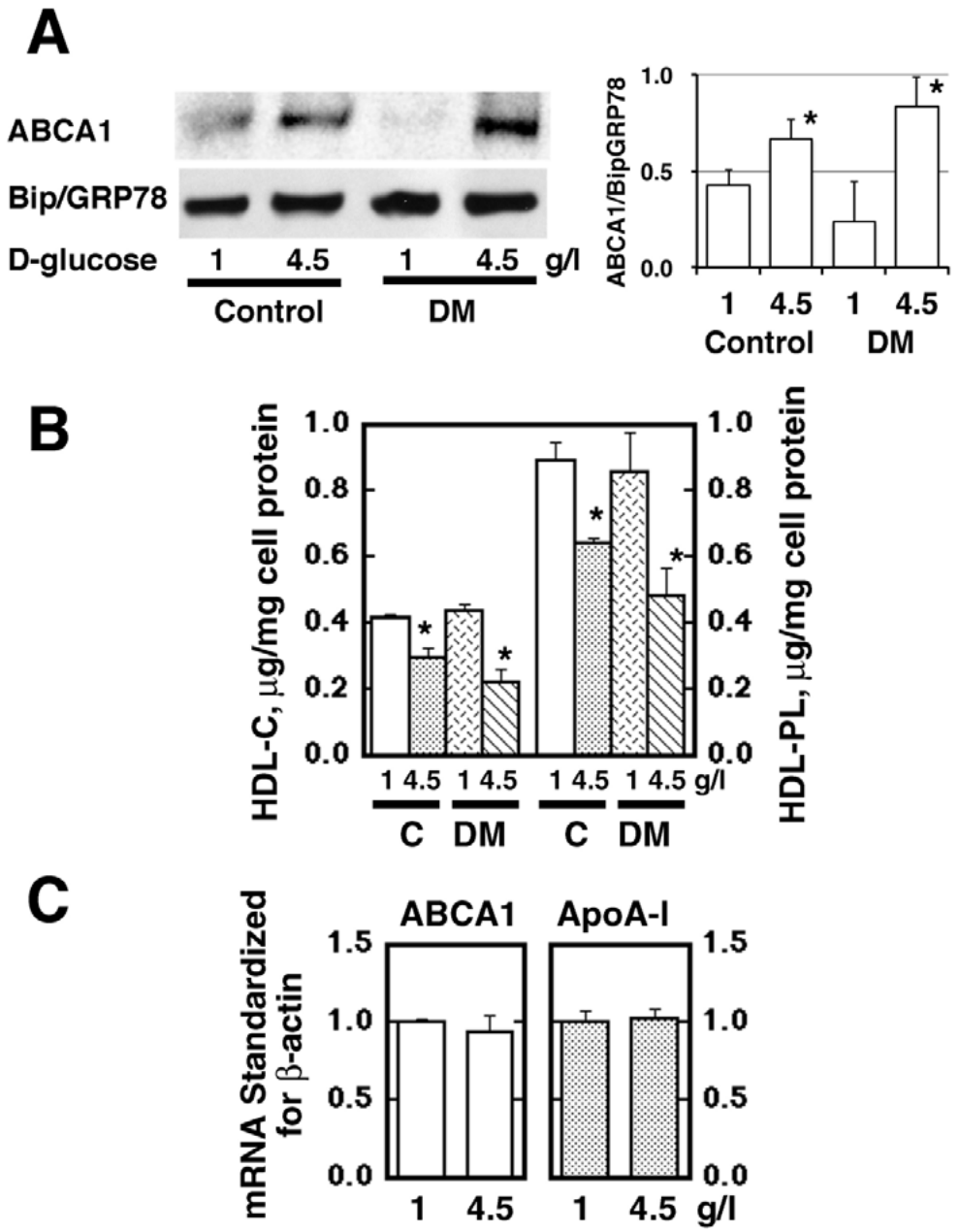

Fig. 2. Expression of ABCA1 and HDL biogenesis in primary hepatocytes isolated from the control and diabetic mice. A: ABCA1 protein analysis by Western blotting. Hepatocytes were prepared from the control and diabetic mice (DM) in high D-glucose media (4.5 g/l) and then cultured in low (1 g/l) and high (4.5 g/l) D-glucose media for 16 h. ABCA1 in the membrane fraction was analyzed in Western blotting using a specific antibody. The right panel shows quantitated results ($n=5$) based on photo intensity of the blotted bands standardized by that of a loading control Bip/GRP78, which unlike the finding *in vivo* (Fig. 1B) stands constant against change in glucose concentration in the culture medium so much as integrin $\beta 1$ and β-actin (data not shown). B: Release of HDL from the primary hepatocytes described above during the 16-h incubation in low or high D-glucose concentrations (1 and 4.5 g/l). Cholesterol and choline-phospholipid were measured for the HDL fraction isolated by ultracentrifugation. C: Expression of ABCA1 mRNA in the primary hepatocytes defined as above. Data represent mean ± SD for three samples. Asterisks indicate significant difference of high glucose (4.5 g/l) from low glucose (1 g/l) by $p<0.05$.

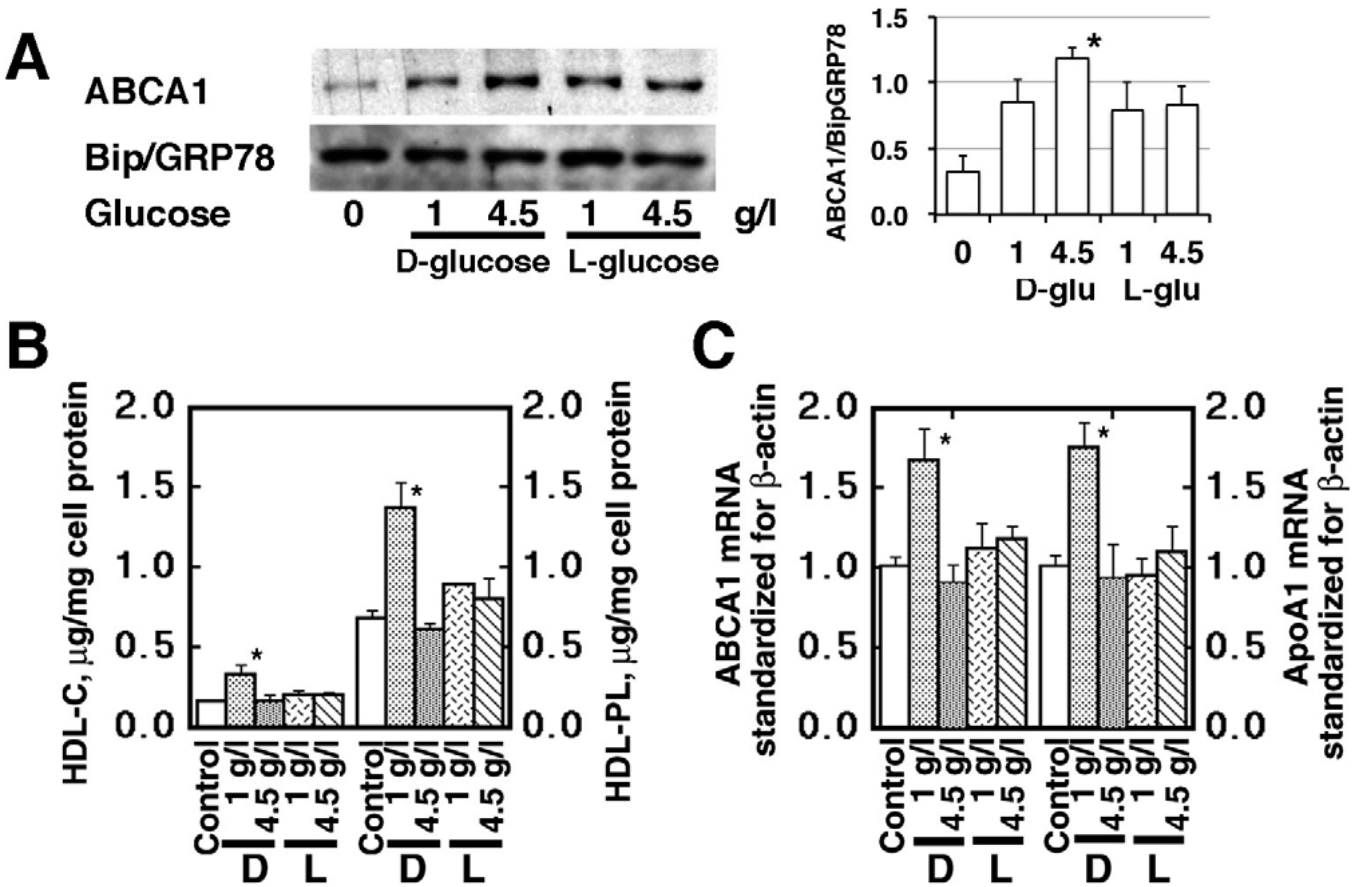

Fig. 3. Expression of ABCA1 and biogenesis of HDL in HepG2 cells. A: ABCA1 protein analysis by Western blotting. HepG2 cells were cultured in the medium containing basic D-glucose concentration of 1 g/l and with additional low or high D-glucose and with additional low and high L-glucose (1 and 4.5 g/l, respectively) for 16 h. ABCA1 in the membrane fraction was analyzed by Western blotting. The bands were quantitated and standardized by Bip/GRP78 ($n=3$). B: Release of HDL from HepG2 cells described above during the 16-h incubation as defined above. Cholesterol and choline-phospholipid were measured for the HDL fraction isolated by ultracentrifugation. C: Expression of ABCA1 mRNA in HepG2 cells defined as above. Data represent mean ± SD for three samples. Asterisks indicate significant increase of high additional D-glucose (4.5 g/l) from low (1 g/l) in Panel A and that of 1 g/l additional D-glucose from others in Panels C and D by $p<0.05$.

Finally, conformation of apoA-I in aqueous solution was examined by circular dichroism. No significant change was identified in high concentration of glucose (**Supplementary Fig. 2**).

Discussion

DM (both type I and type II) is a strong risk factor for atherosclerotic vascular diseases such as coronary heart disease, stroke, and peripheral arterial disease[42, 43]. Prolonged exposure to hyperglycemia is thought to be one of the primary casual factors of pathogenesis of various diabetic complications including atherosclerosis[44-46]. Although the mechanism for hyperglycemia to develop atherosclerosis is unclear[5], it is believed to induce various pathological changes in vascular tissues that potentially promote atherosclerosis[6-8]. One of the findings among those is that plasma HDL concentration, a strong negative risk factor of atherosclerosis, tends to be decreased in diabetic patients[9, 10]. We thus investigated the relationship of hyperglycemia to reduction of HDL.

The findings here are summarized as (1) STZ-induced diabetic animals, a model for primary insulin deficiency diabetes and known for increased atherosclerosis[35], had plasma HDL significantly decreased (**Table 1** and **Supplementary Fig. 1**); (2) in contrast, ABCA1, a key factor for HDL biogenesis, increased in the liver without apparent increase of its mRNA (**Fig. 1**); (3) the findings were reproduced with the primary hepatocytes from these mice and with human hepatoma cell line cells HepG2 cultured in high D-glucose concentration for the decrease of HDL biogenesis despite the increase of ABCA1. The primary

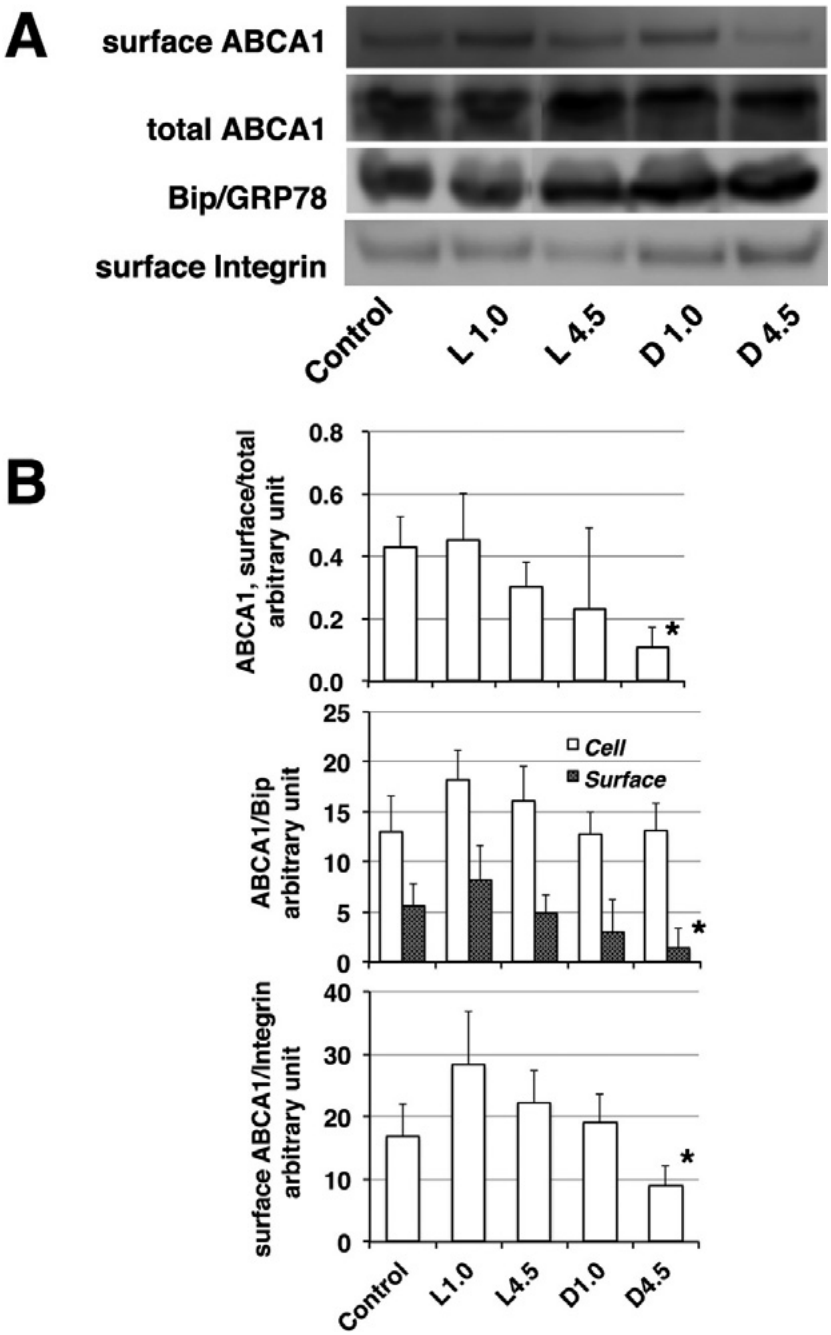

Fig. 4. Surface ABCA1 in HepG2 cells probed by protein biotinylation. HepG2 cells were prepared by incubating in the medium with additional low or high D-glucose and L-glucose (1 and 4.5 g/l, respectively) for 16 h. The cell surface proteins were labeled with biotin was precipitated by avidin beads for further immunoblotting analysis with antibodies against ABCA1 and integrin $\beta 1$. A: Typical data set of immunoblot analysis. B: Surface ABCA1 quantified by scanning density of the bands and standardized for total cellular ABCA1, Bip/GRP78, and surface integrin, for three samples as average ± SE. Asterisks indicate $p < 0.05$ from the control. L1 and L4.5, L-glucose 1 and 4.5 g/l; D1 and D4.5, D-glucose 1 and 4.5 g/l.

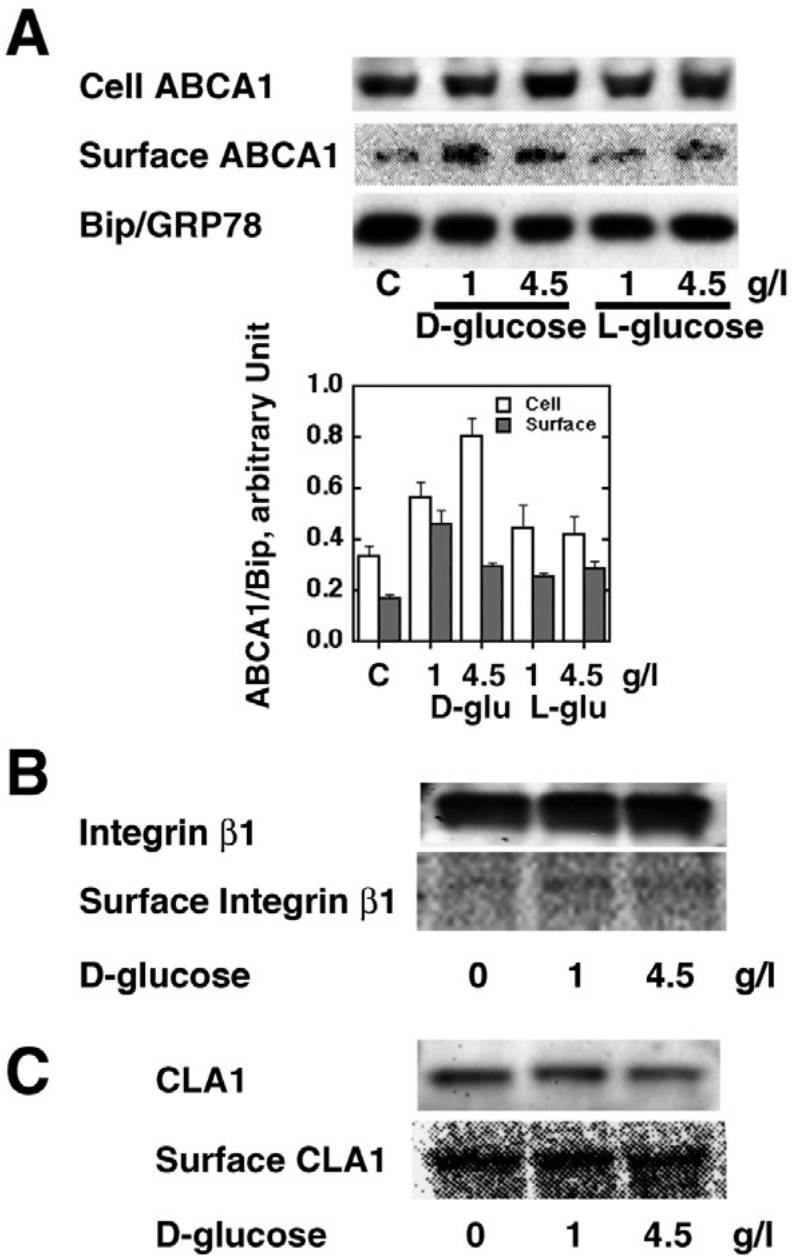

Fig. 5. Surface ABCA1 in HepG2 cells probed by ^{125}I labeling. HepG2 cells were prepared by incubating in the medium with basic (1 g/l) glucose and additional with low or high D-glucose and with additional low and high L-glucose (1 g/l and 4.5 g/l, respectively) for 16 h. Surface proteins were labeled with ^{125}I, and specific membrane proteins were precipitated using protein A-agarose-bound specific antibodies against ABCA1 (A), integrin β1 (B), and CLA-1 (C). The ratio of surface/cell ABCA1 significantly decreased in D-glucose 4.5 g/l ($p < 0.05$). Precipitated membrane proteins were analyzed by SDS-PAGE, and radioactivity in the gel was analyzed by a BAS-1800II Fujifilm. The inset graph to panel A represents the results of digital scanning of the three gels. Cellular ABCA1 and surface ABCA1 were standardized for Bip/GRP78, respectively. D-glu and L-glu indicate D-glucose and L-glucose, respectively.

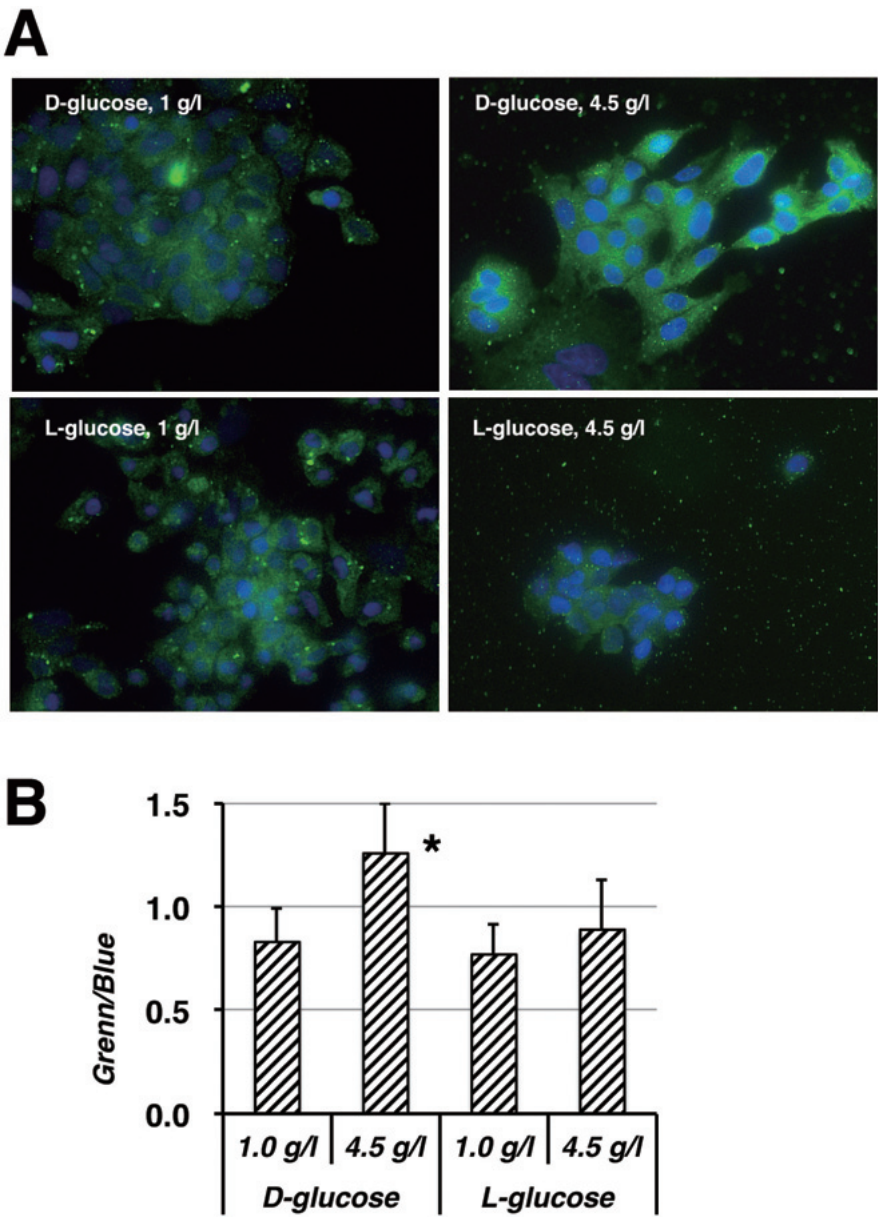

Fig. 6. Fluorescent staining of ABCA1 in Hep G2 cells. A: Typical profiles of the cells cultured with additional D- and L-glucose at the concentrations of 1 and 4.5 g/l. B: Quantitation of the ratio of green/blue photo intensity for an indicator of ABCA1 protein content in cells. The data are based on the measurement of approximately 1000 cells for each condition. Asterisk indicates significance with $p < 0.01$ from others.

hepatocytes transferred to normal culture environment, regardless of control and diabetic, showed no difference in these parameters (**Fig. 2**); (4) cell surface ABCA1 decreased in HepG2 cells cultured in high D-glucose condition despite the increase of total cellular ABCA1 (**Fig. 4, 5**, and **6**); and (5) the rate of ABCA1 decay was retarded in the primary hepatocytes and HepG2 cells exposed to the high D-glucose condition, being consistent with the findings above (**Fig. 7**).

The results indicate that exposure of hepatocytes to high glucose concentration causes reduction of hepatic HDL biogenesis due to decrease of cell surface ABCA1 in the hepatocytes. This decrease in surface ABCA1 seemed not because of enhanced regulatory degradation of ABCA1 that is primarily carried out by calpain after internalization of ABCA1 from the

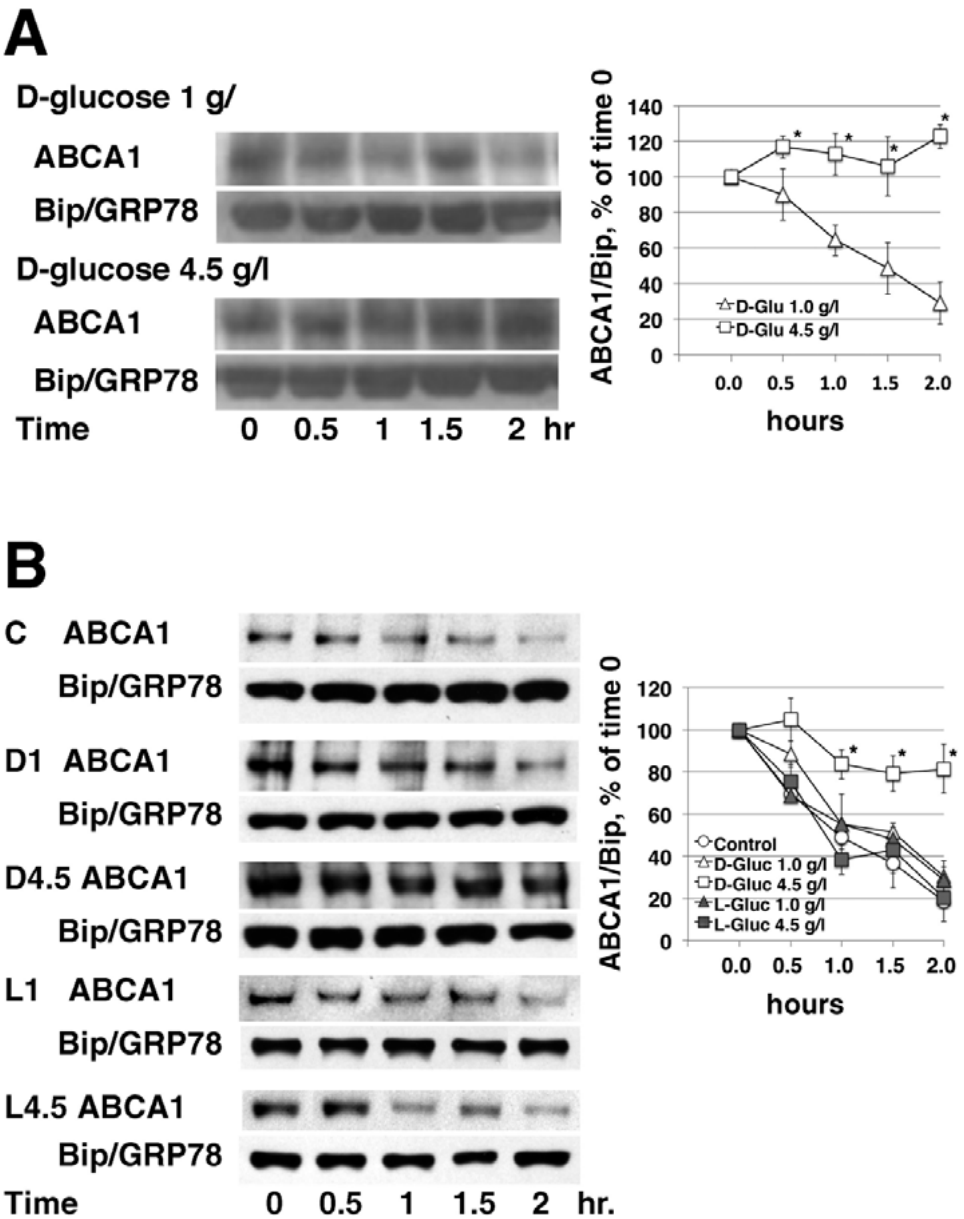

Fig. 7. Decay of ABCA1 in hepatocytes after adding cycloheximide in the low and high glucose medium. Experimental conditions are described in the method section. A: Decay of ABCA1 in the primary hepatocytes isolated from C57BL/6 mice in the medium containing D-glucose at low and high concentrations (1 and 4.5 g/l). B: Decay of ABCA1 in HepG2 cells in the medium with basic (1 g/l) glucose and with additional low or high D-glucose and low and high L-glucose (1 and 4.5 g/l, labeled as D1, D4.5, L1, and L4.5, respectively) for 16 h. The inset graphs represent the results of digital scanning of the three gels. ABCA1 was standardized for Bip/GRP78 and expressed as percent of the value at time zero for each series of the experiment. Asterisks indicate significance of high glucose condition from others.

plasma membrane. Total cellular ABCA1 increased while the surface ABCA1 decreased, so that localization of ABCA1 to the plasma membrane was decreased in this condition, and therefore the rate of ABCA1 degradation was decreased seemingly secondarily to this decrease of surface ABCA1. This is consistent with the findings that cell surface ABCA1 is responsible for the HDL biogenesis reaction with extracellular helical apolipoproteins[29-31, 47-50]. This must be also true in HDL biogenesis in hepatocytes where endoge-

nously secreted apoA-I should interact with cell surface ABCA1 in an autocrine manner[25, 51].

The findings lead to the speculation that high glucose environment impairs the traffic of synthesized ABCA1 molecules to the cell surface, causing its intracellular accumulation and decrease of cell surface ABCA1. The observation by ABCA1 immunostaining was in fact consistent with this hypothesis (**Fig. 6**). The underlying mechanism is still to be investigated. The mechanism for hyperglycemic condition to interfere with intracellular ABCA1 metabolism is still unknown at this stage. The findings are unlikely due to nonspecific physicochemical effect of hyperglycemia since L-glucose was ineffective.

There have been substantial inconsistencies among previous reports about the effects of high glucose on HDL metabolism. Decrease of plasma HDL was not common observation in the STZ-diabetic animals. Some report rather increase of HDL using younger mice or rats[52, 53]. Increase of ABCA1 mRNA and its reporter gene activity by high glucose were also reported in HepG2 cells[54]. The other report demonstrated decrease of plasma HDL in the STZ-diabetic mice with decrease of ABCA1 mRNA in their liver, as well as in HepG2 and RAW264 cells exposed to high glucose[55]. Decrease of ABCA1-mediated HDL biogenesis was indicated as the effects of advanced glycation end products[56]. No rational interpretation has been proposed for these different observations. Our findings are not consistent with all of these previous observations. We have currently no appropriate interpretation for these discrepancies.

STZ-induced diabetes consolidates a model for type I diabetes or IDDM, in which lack of insulin action and following hyperglycemia are primary pathological statuses. They develop atherosclerosis, and hyperglycemia is one of the potential risks[35]. STZ-treated nondiabetic mice did not show low HDL (**Table 1**) so that reduction of HDL was not likely due to STZ itself but to the diabetic condition caused by STZ. Hepatocytes isolated from the control and the diabetic mice both exhibited similar behavior of ABCA1 expression and HDL biogenesis with respect to response to glucose concentration in the medium. This finding indicates that the cause of low HDL production and apparent increase of ABCA1 is not due to preconditioning of the hepatocytes *in vivo* in profound metabolic disorder in the insulin deficiency but rather due to the direct effect of high glucose. The lack of the effect by the same concentration of L-glucose indicates that this is not a simple physicochemical effect such as high osmotic pressure. An important proposal was made for the effects of high glucose through enzymatic glycation of functional cell proteins[57], which may be consistent with the current finding.

Low HDL could also be caused by its accelerated catabolism by the factors such as SRB1 or the human equivalent protein CLA-1 expressed predominantly in the liver[58, 59], but CLA-1 in HepG2 cell surface was not changed by glucose (**Fig. 5C**), and the massage of SRB1 was not changed by high glucose in mouse hepatocytes both *in vivo* and *in vitro* (data not shown). Decrease of HDL biogenesis was unlikely caused by conformational change of apoA-I induced by exposing to high glucose concentration (**Supplementary Fig. 2**).

The limits of the current study are (1) primary hepatocytes, and HepG2 cells may not fairly represent the conditions of the hepatocytes *in vivo* despite that the findings in the cultured cells were consistent with and supported the *in vivo* findings and (2) minimum glucose concentration (1 g/l) was required for survival of the hepatocytes in culture for the duration of the experiments so that the influence of this glucose level could not be eliminated. However, the findings still suggested that the results were not due to mere physical effect of high glucose concentration such as increase of osmotic pressure.

Plasma glucose concentration thus could be an important regulator in maintaining plasma HDL level through influencing localization/traffic of ABCA1 in hepatocytes. The present findings may support the view that direct reduction of blood glucose level by renal SGLT2 inhibitors[11] is beneficial for reduction of a diabetic cardiovascular risk[12].

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Clinical Interest and Disclosures

None.

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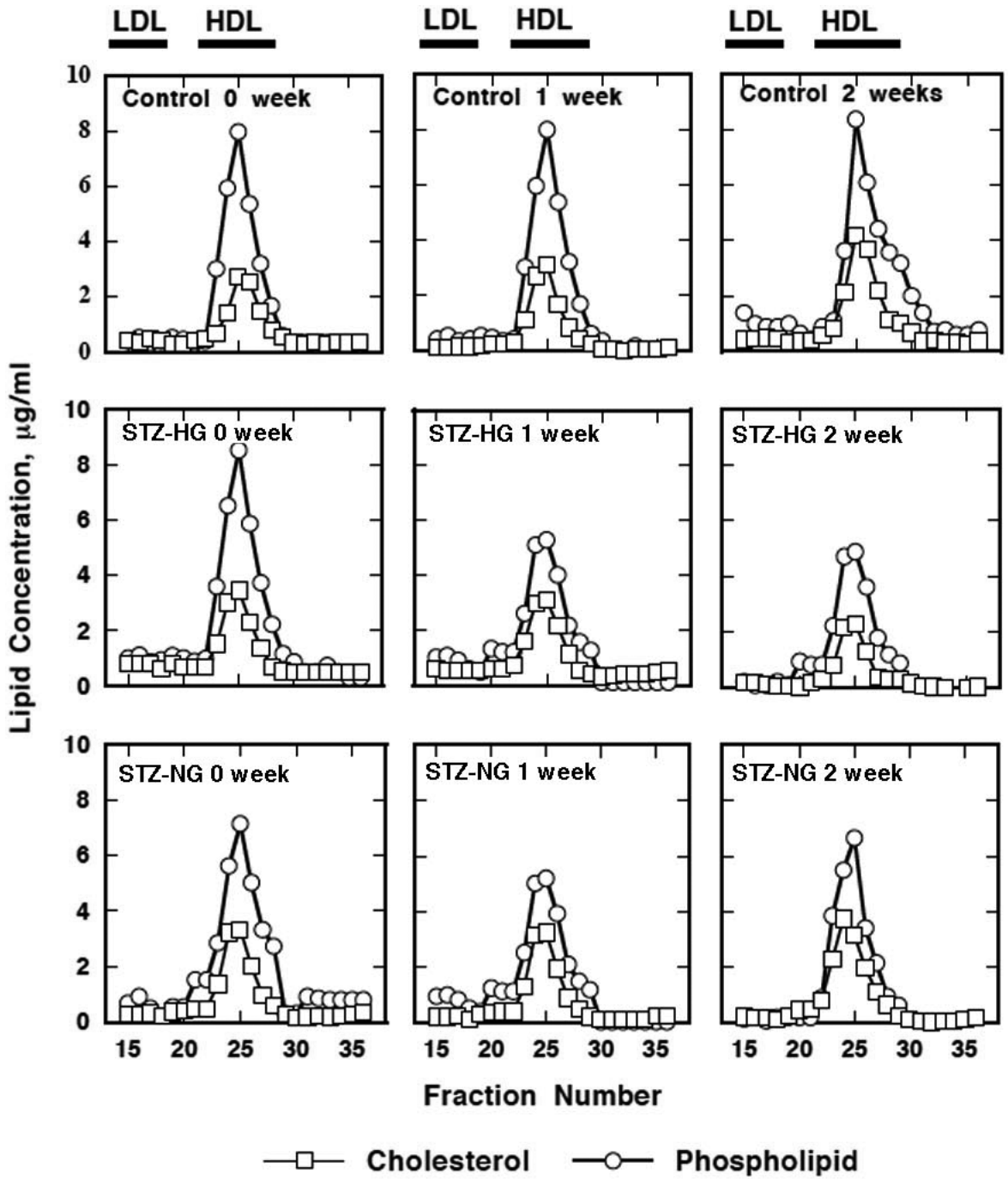
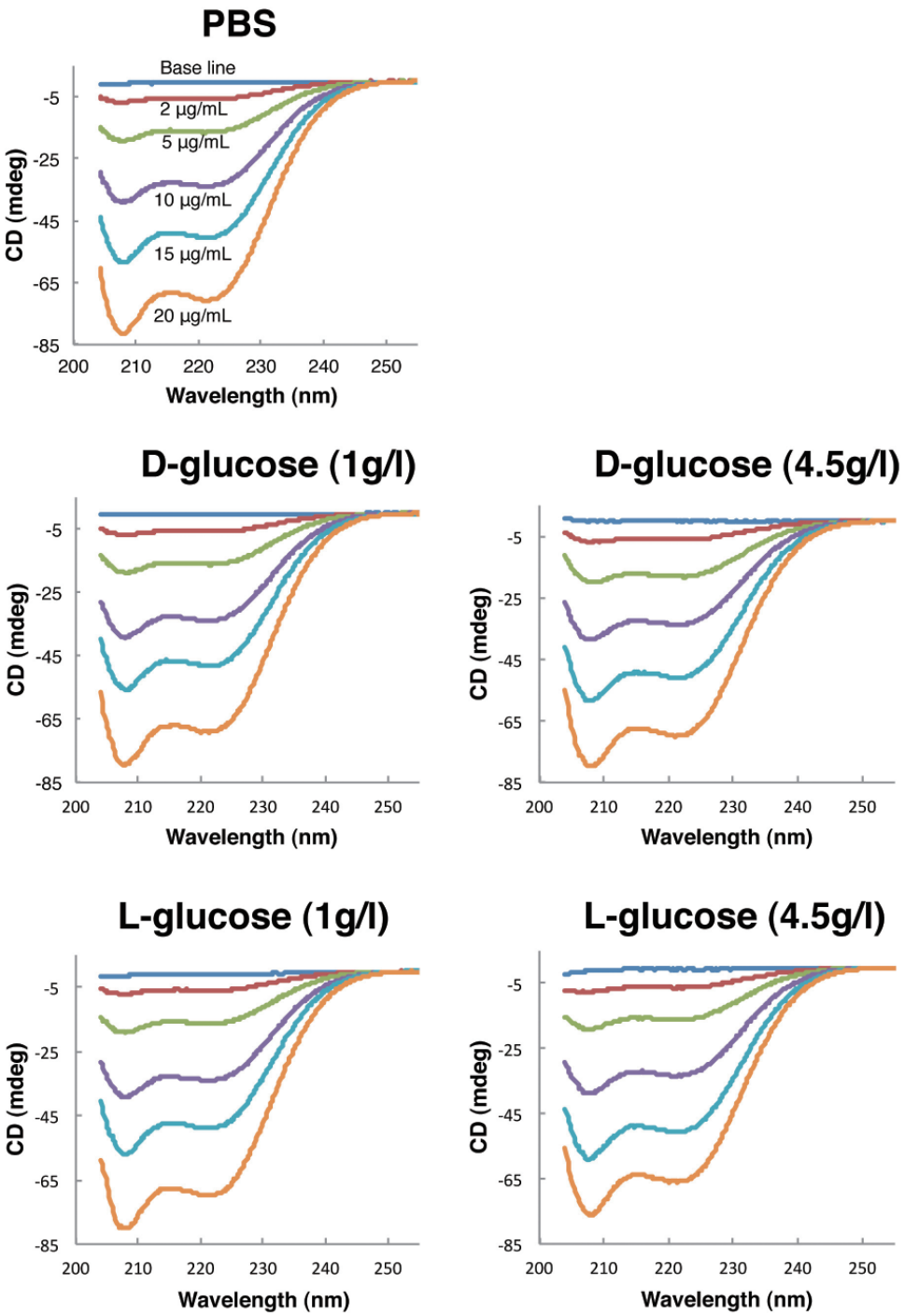


Supplementary Fig. 1. Lipoprotein profiles of streptozotocin (STZ)-treated hyperglycemic (STZ-HG) and normoglycemic (STZ-NG) mice. Mice blood was collected from caudal vein into EDTA-coated microtubes, and plasma was obtained by centrifugation. Mice plasma, 5 μl, was analyzed by gel permeation column, Superose™ 6 Tricorn™ 5/200 GL (207×5 mm, GE Healthcare Life Sciences) in PBS at 0.3 ml/min. The eluent was collected by every 20-s period, 100 μl. Total cholesterol and phospholipid in each fraction were measured using colorimetric enzymatic detection reagents Detaminer L (Kyowa Medics, Japan) with a miniscale detection method. Bars on the top of figure indicate elution range for human LDL and HDL in this column system. Squares indicate total cholesterol, and circles indicate phospholipid in typical elution profiles.



Supplementary Fig. 2. Circular dichroism spectra of human apolipoprotein A-I in low or high D-glucose and L-glucose. Human apolipoprotein A-I was dissolved in PBS, containing 1 and 4.5 g/l D-glucose and 1 and 4.5 g/l L-glucose. The solution was preincubated in 37℃ water bath at least 30 min before transfer into an ES grade silica glass cuvette, T-31-ES10 (TOSOH Quartz Corporation, Japan). Circular dichroism spectrum was taken in a JASCO Spectropolarimerter J-725 equipped with a temperature control unit. Measurement was performed at 37℃, 100 nm/min, and response 0.5 s, and 15 detection data of each sample were integrated by Spectra Manager application version 1.53.07. In PBS (baseline in blue), 2 (red), 5 (green), 10 (violet), 15 (turquoise), and 20 μg/ml (orange) apolipoprotein A-I exhibited concentration-dependent negative CD strength shift. The identical assays performed in the presence of D-glucose or L-glucose at the indicated concentrations are shown in the panels as indicated. No appreciable difference was observed in the CD spectra by presence of glucose at the conditions used.