Glucose-induced activation of GLUT2 gene expression is mediated by SREBP-1c in the hepatocytes

Seung-Soon Im

Department of Medical Science
The Graduate School, Yonsei University

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Directed by Professor **Yong-Ho Ahn**

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This certifies that the Doctoral Dissertation of Seung-Soon Im is approved.

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Thesis Supervisor : Yong-Ho Ahn

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Kyung-Sup Kim

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Bong-Soo Cha

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Min-Goo Lee

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Young-Nyun Park

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‘The LORD is my shepherd, I shall not be in want’

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ABSTRACT

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Glucose transporter type 2 isoform (GLUT2) is mainly expressed in the liver, β-cells of the pancreas, and the basolateral membrane of kidney proximal tubules and plays an important role in glucose homeostasis in living organisms. The transcription of the GLUT2 gene is known to be upregulated in the liver during postprandial hyperglycemic states or in type 2 diabetes. However, a molecular mechanism by which glucose activates GLUT2 gene expression is not known.

In this study, we report evidence that sterol response element binding protein (SREBP)-1c plays a key role in glucose-stimulated GLUT2 gene expression. The GLUT2 promoter reporter is activated by SREBP-1c and the activation is inhibited by

a dominant-negative form of SREBP-1c (SREBP-1c DN). Adenoviral expression of SREBP-1c DN suppressed glucose-stimulated GLUT2 mRNA level in primary hepatocytes. An electrophoretic mobility shift assay and mutational analysis of the GLUT2 promoter revealed that SREBP-1c binds to the –84/-76 region of the GLUT2 promoter. Chromatin immunoprecipitation revealed that the binding of SREBP-1c to the -84/-76 region was increased by glucose concentration in a dose-dependent fashion. These results indicate that SREBP-1c mediates glucose-stimulated GLUT2 gene expression in hepatocytes.

Key Words : Liver, glucose transporter type 2 (GLUT2), Sterol response element binding protein-1c (SREBP-1c), glucose

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Ⅰ. INTRODUCTION

ADD1/SREBP-1c (adipocyte determination and differentiation dependent factor 1/sterol response element binding protein-1c) is a *trans*-acting factor that regulates transcription of many genes involved in cholesterol and fatty acid synthesis[1-6]. To date, three isoforms of SREBP have been identified. SREBP-1a and -1c are derived from a single gene and use different transcription initiation sites[7]. SREBP-2 is transcribed from a separate gene[8] and shows 50% sequence homology to the SREBP-1c isoform. SREBP-2 plays a critical role in the regulation of genes of cholesterol biosynthesis

whereas SREBP-1c regulates lipogenesis genes in liver[9].

The relative contribution of insulin[10-14] or glucose[15-17] to the regulation of specific genes is known to be different. In non-fasted mice, the level of SREBP-1c transcripts in the liver is considerably more abundant than the SREBP-1a transcripts[3]. Fasting decreases the amount of SREBP-1c transcripts by 60%, and refeeding increases the level by 3.8-fold[3]. In contrast, the fasting/refeeding protocol causes only minor changes in the amount of SREBP-1a transcripts[18]. In insulin resistance animals, such as *ob/ob* mice where insulin signaling is known to be impaired[19], or other insulin signaling system knockout animals[20], overexpression of SREBP-1c in the liver was reported[12]. These reports suggested that SREBP-1c induction could also occur by glucose.

The facilitative glucose transporters (GLUTs) are a group of highly related membrane proteins that share significant sequence homology between isoforms[21]. GLUT2 is known to transport glucose across the hepatic plasma membrane in a bi-directional manner[22, 23]. GLUT2 was upregulated by glucose, like pyruvate kinase (PK) and fatty acid synthase[24]. In diabetic states, GLUT2 in the liver and basolateral membrane of renal proximal tubules is known to be upregulated, indicating that hyperglycemia may play a positive role in its gene regulation[25]. And in streptozotocin-induced diabetic rats, where insulin was depleted *in vivo*[10], SREBP-1c expression was increased in liver. These data lead us to postulate that GLUT2 upregulation in the liver could be due to the activation of the gene by SREBP-1c.

From this background, we have attempted to identify the SREBP-1c response

element (SRE) in the promoter region of GLUT2 and demonstrate that glucose increases the binding of SREBP-1c on the GLUT2 promoter, resulting in the activation of GLUT2 transcription.

Ⅱ. MATERILAS AND METHODS

1. Materials

All the reagents for cell culture, such as media, fetal bovine serum, antibiotics, and LipofectAMINE PLUS were purchased from GibcoBRL, Rockville, MD, USA and Life Technologies, Gaithersburg, MD, USA. [γ-^{32}P] ATP, [α-^{32}P] dCTP, Rediprime labeling kits, and a rapid hybridization solution were purchased from Amersham Biosciences, Piscataway, NJ.

2. Preparation of recombinant SREBP-1 and SREBP-1 antibody

Recombinant SREBP-1 was prepared as described[5]. Briefly, recombinant human SREBP-1 were expressed in *E`scherichia coli* BL21(DE3)pLysS. SREBP-1 expression vector, pET-SREBP-1a was generated by inserting the cDNA fragments from pCSA10, between the EcoRI and SalI sites of pET-21a (Novagen, Darmstadt, Germany) and used for transformation. The recombinant SREBP-1 was induced for 4 h with 1 mM isopropyl-β-D-thiogalactopyranoside. The recombinant proteins containing amino-terminal T7 and carboxyl-terminal polyhistidine (His_6) tag were purified to homogeneity by Ni-NTA-agarose (QIAGEN, Valencia, CA, USA) chromatography. The purity and concentration of the recombinant proteins were verified by SDS-polyacrylamide gel electorphoresis followed by Coomassie Brilliant Blue staining. Polyclonal antibody against the recombinant SREBP-1 was prepared by standard

protocol which was prepared as described previously[26]. One mg of recombinant SREBP-1 protein was suspended in 1 ml of PBS and emulsified with 1 ㎖ of Freund's complete adjuvant (Sigma-Aldrich Co., St. Louis, MO). The emulsified solution was subcutaneously injected into the back of New Zealand white rabbit. After 3 weeks, the booster injection was administered with the same amount and volume of antigen emulsified with Freund's incomplete adjuvant (Sigma-Aldrich Co). The booster injections were repeated three times with 3 weeks intervals. At 10 days after the final booster injection, the blood was collected by the heart puncture under the anesthesia using 50 mg/kg of ketamine hydrochloride and 5 mg/kg of xylazine hydrochloride. The collected blood was incubated in 37℃ for 1 h to complete coagulation and the serum was isolated after centrifugation at 10,000 x g for 10 min.

3. Animals and Treatments

Male ICR mice (7 weeks) were fed a fat-free, high carbohydrate diet containing 82% (w/w) carbohydrates (74% starch, 8% sucrose), 18% (w/w) casein, 1% vitamin mix and 4% (w/w) mineral mix and then fasted for 24 h. All the materials for the diet were purchased from Harlem Teklad Co., Madison, WI, USA.

4. Primary hepatocytes preparation from mouse liver

Hepatocytes were isolated from a male ICR mouse (7 weeks) by the collagenase perfusion method[27]. Dissociation into individual hepatocytes was performed in

Dulbecco's modified Eagles' medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% heat inactivated fetal bovine serum, 100 nM insulin, 10 μM dexamethasone, 25 mM glucose, 100 unit/ml penicillin G and 100 μg/㎖ streptomycin. For each hepatocyte preparation, cell viability was estimated by the exclusion of trypan blue.

5. Isolation of total RNA and northern blot analysis

Total cellular RNA was extracted from primary hepatocytes using the TRIzol reagent method (Life Technologies)[28] and prepared according to the manufacturer's protocol. Twenty μg of each sample was denatured and subjected to electrophoresis in a 0.9% denaturing formaldehyde-agarose gel and transferred to a nylon membrane. The membranes were hybridized with the probe for 3 h at 65 °C with Rapid-Hybrid buffer (Amersham Biosciences). After hybridization, the membrane was washed twice with a high salt washing buffer (0.1% SDS, 2x SSC) at room temperature for 30 min, followed by a low salt washing buffer (0.1% SDS, 0.2x SSC) at 65 ℃ for 15 min. The membrane was exposed to Kodak BioMax film using an intensifying screen at –70 °C.

6. RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from primary hepatocytes using TRIzol reagent according to the manufacturer's protocol (Life Technologies). For RT-PCR, the first strand of cDNA was synthesized from 3 ㎍ of total RNA using random hexamers and Superscript II

reverse transcriptase (Life Technologies). One microliter of the reverse transcription reaction mixture was amplified with primers specific for GLUT2 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) in a total volume of 50 $\mu\ell$. Linearity of the PCR was tested with amplification cycles between 25 and 30. According to the test amplification profile, samples were amplified using the following parameters: 94°C for 30 s, 56°C for 30 s, and 72°C for 28 s. G3PDH was used as an internal control for quality and quantity of RNA. The PCR primers used were as follows: GLUT2 sense, 5'-GGCTAATTTCAGGACTGGTT-3'; GLUT2 antisense, 5'-TTTCTTTGCCCTGACT TCCT-3'; G3PDH sense, 5'-ACCACAGTCCATGCCATCAC-3'; G3PDH antisense, 5'-TCCACCACCCTGTTGCTGTA-3'.

7. Quantitative real-time PCR

Primers for GLUT2, SREBP-1a, SREBP-1c, SREBP-2 and α-tubulin genes were designed using MacVector software (Accelrys, San Diego, CA, USA). The PCR reaction mixture was performed in 10 $\mu\ell$ (final volume) in glass capillary tubes (Roche Diagnostics, GmbH, Germany). The PCR mixture contained 1 $\mu\ell$ of 100 ng cDNA template, 1 $\mu\ell$ of a commercial ready-to-use mixture of LightCycler-DNA Fast Start master hybridization probes (Roche Diagnostics), 5 mM $MgCl_2$ (final concentration), and primers and probes at final concentrations of 5 μM and 1 μM, respectively. Real-time PCR was carried out using a LightCycler instrument (Roche Diagnostics). The conditions for thermal cycling were as follows: initial denaturation

for 10 min, followed by 40 amplification cycles at 95 °C for 10 s, 58 °C for 5 s, and 72 °C for 14 s. Fluorescence was measured at 640 nm (F2 channel) at the end of each annealing phase. The amplification was followed by a melting program, which started at 45 °C for 15 s and then increased to 95 °C by 0.1 °C/s, with the fluorescence signal continuously monitored on-line. Primers and probes were as follows: GLUT2 sense, 5'-GGCTAATTTCAGGACTGGTT-3'; antisense, 5'-TTTCTTTGCCCTGACTTCCT-3'; SREBP-1a sense, 5'-GGCCGAGATGTGCGAACT-3'; antisense, 5'-TTGTTGATGAG CTGGAGCATGT-3'; SREBP-1c sense, 5'-GGAGCCATGGATTGCACATT-3'; anti-sense, 5'-GGCCCGGGAAGTCACTGT-3'; SREBP-2 sense, 5'-GCGTTCTGGAGA CCATGGA-3'; antisense, 5'-CACAAGTTGCTCTGAAAACAAATCA-3'; α-tubulin sense, 5'-CTCGCATCCACTTCCCTC-3'; and antisense, 5'-ATGCCCTCACCCACGT AC-3'.

8. Construction of GLUT2 promoter luciferase plasmids

The GLUT2 promoter fragments of -1112/+1 bp were PCR-amplified from mouse genomic DNA and subcloned into a pGLM vector and mutated at the E-boxes upstream of the multiple cloning site of the pGL3 basic vector. 5' serial deletions of mouse GLUT2 promoter reporter were constructed by amplifying the regions of -891/+1, -389/+1, -286/+1, -116/+1 and -57/+1 bp, respectively. SRE mutant construct pmGLUT2d-389m was generated from pmGLUT2d-389 with the mutagenic oligonuleotides (30 mer) using *pfu* polymerase. The sequences of all constructs were

confirmed using a T7 sequencing kit (Amersham Biosciences). All transfection plasmids were prepared with the Qiagen Plasmid Midi Kit (QIAGEN).

9. Transient transfection assay

Alexander cell lines (American Type Culture Collection number CRL-8024), which are human epithelial hepatoma cell lines, were plated at a density of $2x10^5$ cells/35-mm dish. On the following day, 0.5 μg of each construct of the GLUT2 promoter, 0.1 μg of pCMV-β-galactosidase, with or without 0.1 μg of SREBP-1c, and 0.5 μg of SREBP-1 dominant negative (DN) expression vector were mixed. Transient transfection and luciferase assays were performed as described previously[29]. Total protein concentrations of the lysates were determined by the Bradford method[30]. Luciferase activities were normalized by the amount of total protein. Each transfection was performed in triplicate and repeated three to five times.

10. Electrophoretic mobility shift assay (EMSA)

The oligonucleotide probes GLUT2-SRE and GLUT2-SREm (GLUT2-SRE mutant) were labeled as described previously[31]. Ten picomoles of the single stranded sense oligonucleotide were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase and annealed with 50 pmoles of unlabeled antisense oligonucleotides. For competition assay, unlabeled oligonucleotides (100-fold molar excess) were added to the reaction mixture. The oligonucleotide sequences used in EMSA were as follows: GLUT2-SRE

sense, 5'-CCAGGTAGAGTGAGCACTCT-3'; antisense, 5'-AGAGTGCTCACTCTA CCTGG-3'; GLUT2-SREm sense, 5'-CCAGGTAGAG**aac**GCACTCT-3'; antisense, 5'-AGAGTGC**gtt**CTCTACCTGG-3'; mLDLR-SRE sense, 5'-TTGAAAATCACCCCATT GCAGACTCCTCCCCGGC-3'; and antisense, 5'-GCCGG GGAGGAGTCTGCAATG GGGTGATTTTCAA-3'. Mutated bases are represented in bold and underlined.

11. Preparation of recombinant adenovirus

Recombinant adenovirus encoding SREBP-1c or dominant negative form of SREBP-1c (SREBP-1c DN) was prepared according to Choi *et al*[32]. Briefly, the respective cDNAs were cloned into pAd-YC2 (5μg) and a rescue vector, pJM17 (5μg) were cotransfected into 293 cells for homologous recombination. After 12 to 15 days, recombinants were screened by PCR using upstream primers derived from the CMV promoter and downstream primers from the bGHp(A) sequence. The recombinants were amplified in 293 cells and purified and isolated using $CsCl_2$ (Sigma-Aldrich Co.). The recombinant adenoviruses were collected, desalted, and titers were determined by the measurement of plaque counts.

12. Treatment of recombinant adenovirus

Null, adenovirus-GFP or adenovirus containing SREBP-1 or SREBP-1c DN were treated according to the method described by Kim *et al*[29]. The primary hepatocytes were incubated for 2 h with an adenovirus containing DMEM at a titer of 5 plaque-

forming units per cell for 2 h at 37 ℃. Culture medium was then replaced by DMEM supplemented with 10% fetal bovine serum. The infected cells were harvested for RNA isolation 24 h after viral infection.

13. Western blot immunostaining

Western blot analysis was performed as previously described[33]. The cell was harvested by the ice-cold PBS (pH 8.0) from primary hepatocytes culture after treating 5 mM to 25 mM glucose for 24 h. Eighty micorgrams of cell lysates were prepared by adding 1X SDS sample loading buffer followed by 20 bursts of sonication (Cycle: 0.5, amplitude 80%) and boiling at 95 °C for 5 min. The protein samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience GmbH, Dassel, Germany). The blotted membranes were immunostained with ani-GLUT2 (1:500; Chemicon, Temecula, CA, USA) and a-tubulin (1:2000; Oncogene Science, Cambridge, MS) as primary antibodies and detected by enhanced chemiluminescence (ECL, Amersham Biosciences).

14. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay protocol was adapted from methods described by Duong *et al*[34]. The primary hepatocytes were fasted in fasting medium composed of 2 mM glutamine, 1 mM sodium pyruvate, 10 mM lactate, 10 nM dexamethasone and 2% FBS for 16 h. The medium was then changed to one which contained 25 mM glucose and/or 100 nM

insulin for 24 h. Cells were washed with ice-cold PBS and cross-linked with 5% formaldehyde in serum-free DMEM for 5 min. The cells were homogenized, pelleted by centrifugation at 2000 rpm for 4 min at 4 °C, and resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0). The lysate was sonicated on ice for 3 min at a setting of cycle 0.5, amplitude 30 and sheared to between 100 and 600 bps. To provide a positive control (input) for each condition, one undiluted aliquot was retained for further processing in parallel with all the other samples at the reversal of the cross-linking step. To reduce nonspecific background, each chromatin sample (1 ㎖) was pre-cleared with 60 ㎕ of protein A/G agarose (Santa Cruz Biotechnology Inc., CA, USA), supplemented with 200 ㎍/㎖ sonicated salmon sperm DNA (Stratagene, La Jolla, CA, USA), and the beads were pelleted. Chromatin complexes in the supernatant were immunoprecipitated overnight at 4 °C using either 30 ㎍ of SREBP-1 antibody or without antibody. Immune complexes were collected with 60 ㎕ of protein A/G agarose including 200 ㎍/㎖ of salmon sperm DNA (Stratagene). Promoter-specific PCR GLUT2 promoter primers were as follows: rGLUT2-sense, 5'-CCATCAATACTCAGCTTCTG-3'; rGLUT2-antisense, 5'-TGTGTGTGTGTGGAATTGTC-3', mFAS-sense, 5'-CAGCCCCGACGCTCATTGG-3'; mFAS-antisense, 5'-GCCCGCCTATCCTTCCACTG-3'.

15. Statistical analysis

All transfection studies were performed in three to five separate experiments, where

triplicate dishes were transfected. The data were represented as a mean ± S.D. Statistical significance was determined by independent two-sample t-tests or paired t-tests between two groups of data sets.

Ⅲ. RESULTS

1. Effect of glucose and insulin on the mRNA level of GLUT2 and SREBPs in primary cultured hepatocytes

In order to observe the effects of glucose and/or insulin on the expression of SREBP-1 and GLUT2, we treated primary cultured hepatocytes with high glucose (25 mM), insulin (100 nM), or both. Northern blot and real time PCR analyses showed that treatment with insulin (100 nM) or glucose (25 mM) increased the SREBP-1 mRNA level (Fig. 1A and 1B). However, GLUT2 expression was not affected by insulin alone but was stimulated by addition of high glucose (Fig. 1B, $*p < 0.001$ untreated vs glucose treated group). These data suggest that glucose is a major determinant in GLUT2 gene upregulation. Real-time PCR of the SREBP-1 isoform revealed that insulin or glucose treatment activated SREBP-1c expression (Fig. 1C), whereas SREBP-1a and SREBP-2 expressions were not affected by both insulin and glucose (Fig. 1D and 1E). Insulin and glucose increased SREBP-1c mRNA level by 7-fold and 4-fold, respectively (Fig. 1C, $*p < 0.005$, untreated vs insulin treated group, $^{\#}p < 0.05$, untreated vs glucose treated group). Insulin and glucose showed additive effect (Fig. 1C, $^{\dagger}p < 0.001$, untreated vs glucose/insulin treated group) in activating SREBP-1c expression.

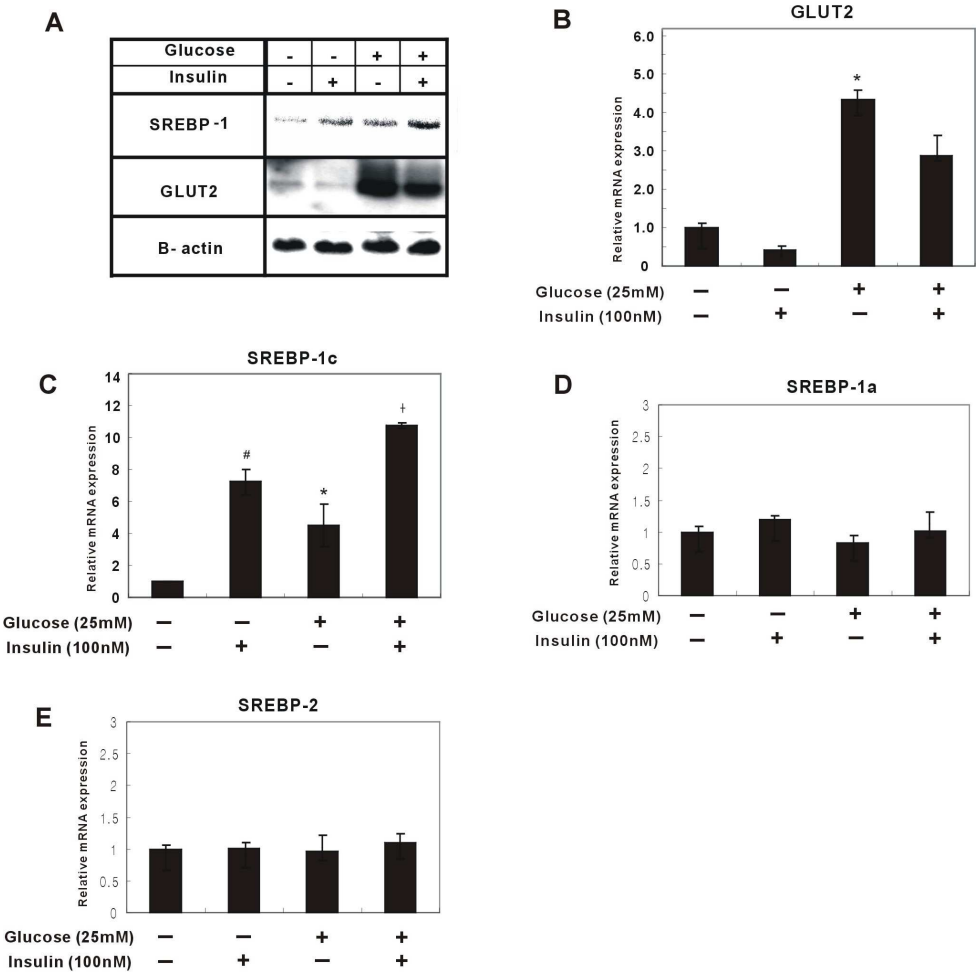

Figure 1. Effect of glucose and insulin on the mRNA level of GLUT2 and SREBPs in primary cultured hepatocytes. A, Hepatocytes isolated from mice were plated for 5 h and cultured further for 16 h in the presence or absence of insulin (100 nM) and glucose (25 mM). Total RNA was extracted from each treatment group as described in "RESEARCH DESIGN AND METHODS". **B-E,** Quantification of hepatic mRNA for GLUT2, SREBP-1c, SREBP-1a and SREBP-2. Total RNA (3 μg) used in reverse transcription experiments were subjected to real-time PCR. The data were quantified as

described in ‘RESEARCH DESIGN AND METHODS’. All mRNA levels were normalized to that of α-tubulin. The results are the means ± S.D. of three independent experiments with triplicate measurements. Fig. 1B, *untreated vs glucose treated group (*$p < 0.001$*), Fig. 1C, *untreated vs insulin treated group (*$p < 0.005$*), $^{\#}$untreated vs glucose treated group (*$p < 0.05$*), †untreated vs glucose/insulin treated group (*$p < 0.001$*)

2. Effect of SREBP isoforms on GLUT2 promoter reporter activity in the Alexander cell lines

As shown in Fig. 2, SREBP-1c activated GLUT2 promoter reporter by 5.2-fold (*$p < 0.001$, untransfected vs SREBP-1c group), whereas SREBP-2 activated the promoter by 2.4-fold ($^{\#}p < 0.05$, untransfected vs SREBP-2 group), suggesting that SREBP-1c acts as more potent activator of GLUT2 promoter than SREBP-2 isoform.

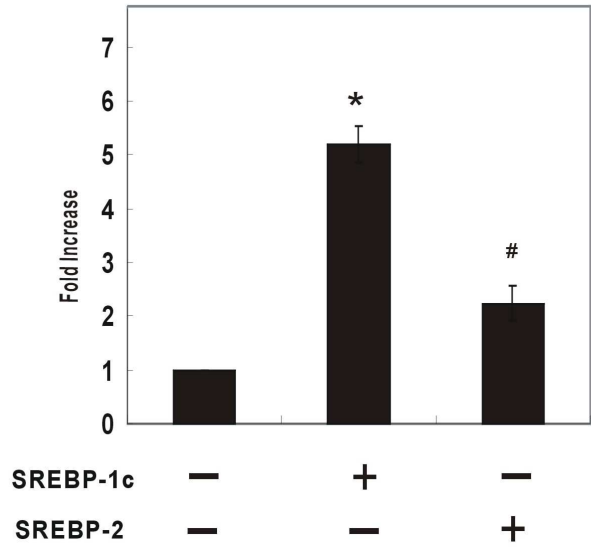

Figure 2. Effect of SREBPs on GLUT2 promoter activity in the Alexander cell lines. pmGT2-1112 promoter reporter was co-transfected with SREBP-1c (100 ng) or SREBP-2 (100 ng) to Alexander cell lines which were plated at a density of 2 x 10^5 cells/35-mm dish. Transient transfection, luciferase assays, and measurement of total protein concentration of the lysates were performed as described in "RESEARCH DESIGN AND METHODS". Luciferase activities were normalized by the amount of total protein. Each transfection was performed in triplicate and repeated three to five times. *Untreated vs SREBP-1 transfected group (*p< 0.001).* #Untreated vs SREBP-2 transfected group *(p < 0.05).*

3. Localization and characterization of SRE in the GLUT2 promoter

To identify a putative SRE in the mouse GLUT2 promoter, serial deletion constructs of the 5'-flanking region of the GLUT2 promoter were prepared (from -1112, -890, -389, -283, -166, and -57 to +1, shown in Fig. 3A) and their responsiveness to SREBP-1c in Alexander cell lines was observed. The SREBP-1c effect was decreased by deleting the bases down to -57 (pmGT2d-57), indicating that a putative SRE could be located between the -166 and -57 bp regions (Fig. 3B, $*p < 0.005$, -166 vs -57 deletion construct). A consensus sequence search suggested that a highly conserved SREBP-1 binding site could be present in this region. To identify an SRE in this region, EMSA was performed. Recombinant SREBP-1[5] bound well with the probe in a dose-dependent manner up to 60 ng (Fig. 3C). The specificity of the SREBP-1c binding was further confirmed by the SRE consensus sequence from the low density lipoprotein receptor (LDLR) promoter[35] (Fig. 3D, lane 3). Mutation introduced at -78/-76 (TGA → aac, SRE-mutant in Fig. 3D) resulted in a loss of SREBP-1 binding (Fig. 3D, lanes 5 and 6). The aac mutant lost its ability to compete the binding of SREBP-1 to the GLUT2-SRE consensus sequence (Fig. 3D, lane 4). Transfection of the putative GLUT2-SRE mutant promoter construct (pmGT2d-389m) lost its responsiveness to SREBP-1c (Fig. 3E, ■). These results indicate that the -84/-76 region acts as a possible SRE regulating GLUT2 gene expression.

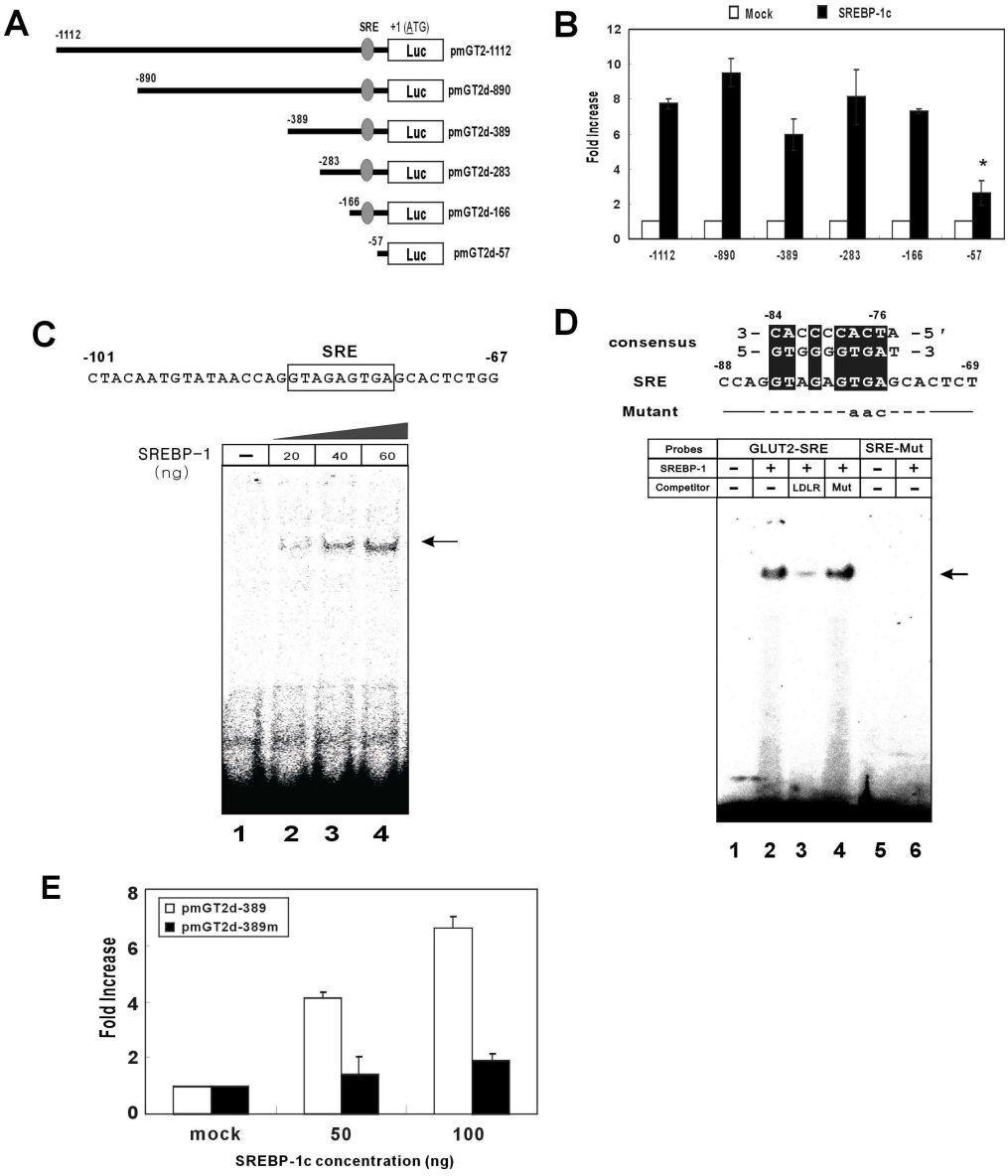

Figure 3. Localization and characterizaion of SRE in the GLUT2 promoter. A, Schematic diagram of serial deletion constructs of GLUT2 promoter reporter to localization of SRE in the GLUT2 promoter. The indicated numbers represent the number of nucleotides from the ATG codon. **B,** Effect of SREBP-1c on the deletion constructs of GLUT2 promoter. The promoter activities were measured by co-

transfecting 100 ng of SREBP-1c expression or empty vectors into Alexander cell lines. The results were normalized by the amount of total protein of lysates, which were determined by the Bradford method and shown as the fold changes of luciferase activities compared to those of the control. Normalized luciferase activities are shown as the means ± S.D. of three independent experiments in triplicate. *-166 vs -57 deletion construct ($p < 0.005$) **C,** Electrophoretic mobility shift assay of GLUT2-SRE. The assay was performed with recombinant SREBP-1 protein in 4% (w/v) non-denaturing polyacrylamide gel. Fifty thousand cpm (0.1 pmole) of ^{32}P-labeled GLUT2 promoter fragments (-101/-67) containing putative SRE was incubated with 20 ng, 40 ng, and 60 ng of recombinant SREBP-1 protein. **D,** Effect of mutation on the SREBP-1 binding to the putative GLUT2-SRE. Site-directed mutation was introduced into the GLUT2-SRE sequence. SRE-mutant was prepared by replacing TGA with aac. Wild type and mutant probes were labeled with [γ-^{32}P] ATP, and EMSA was performed in 4% (w/v) non-denaturing polyacrylamide gel. For these experiments, 30 pmoles of each double-stranded oligonucleotide and 80 ng of SREBP-1 recombinant protein were used. Consensus sequence of SRE reported in the LDLR promoter and mutant oligonucleotides were used as competitors. The DNA-protein complexes are indicated by an arrow. **E,** Effect of mutation on the GLUT2-SRE on the SREBP-1c driven promoter activity. A pSV-SREBP-1c expression vector was co-transfected with pmGT2d-389 or pmGT2d-389m into Alexander cell lines. The luciferase activities were represented as fold changes compared to control group. Values are the means ±S.D. of three independent experiments in triplicate.

4. Effect of a GLUT2-SRE mutation on the glucose-stimulated GLUT2 promoter reporter activity

To confirm that -84/-76 region is a functional SRE in the GLUT2 promoter, the GLUT2 promoter reporters (pmGT2d-389 and pmGT2d-389m) were transfected to Alexander cell lines maintained at 5 mM and 25 mM concentration of glucose. The wild type promoter reporter activity was increased by glucose in a dose-dependent manner (Fig. 4A, $*p < 0.05$, 0 mM vs 25 mM glucose treated group). The GLUT2-SRE mutant construct (pmGT22d-389m) showed decreased promoter reporter activity when compared to wild type promoter (pmGT22d-389, $^{\#}p < 0.05$, wild vs mutant type at 25 mM glucose). Again, the activation of the promoter construct by glucose (25 mM) was suppressed by cotransfection of the SREBP-1c DN (Fig. 4B, $^{\#}p < 0.05$, 25 mM glucose vs 25 mM glucose plus SREBP-1c DN), indicating that GLUT2 promoter activation by glucose is specifically mediated by SREBP-1c.

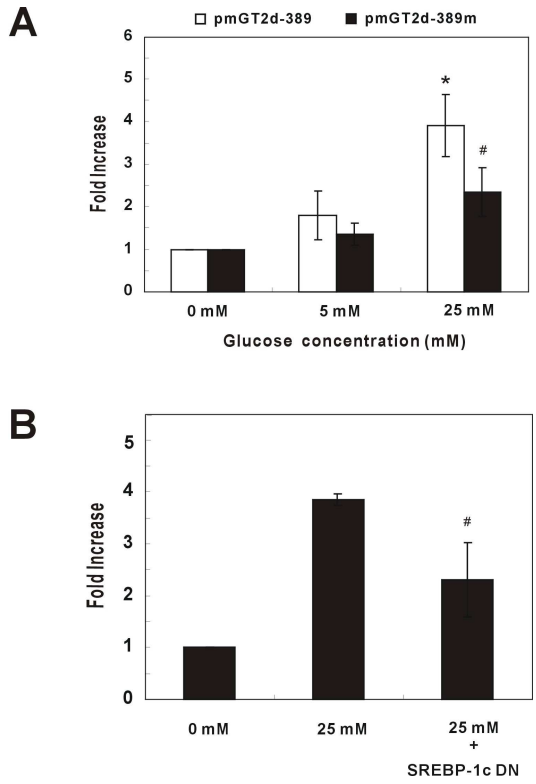

Figure 4. Effect of a GLUT2-SRE mutation on the glucose-stimulated GLUT2 promoter reporter activity. A, Effect of mutation in the putative SRE on glucose mediated GLUT2 promoter activity. A pmGT2d-389 (wild type) or pmGT2d-389m (mutant type) luciferase reporter vector was transfected into mouse primary hepatocytes with the indicated amount of glucose. *Wild type, 0 mM vs 25 mM glucose treated group ($p < 0.05$), #wild vs mutant type at 25 mM glucose ($p < 0.05$, using paired t-test). **B,** Effect of SREBP-1c DN on the glucose-stimulated GLUT2 promoter activity. pmGT2d-1112 and SREBP-1c DN were transfected into primary hepatocytes for 16 h and 25 mM glucose was added to the culture media for 24 h. The luciferase activities were represented as fold changes compared to those of the control group. Values are the means ± S.D. of three independent experiments in triplicate. # 25 mM glucose vs 25 mM glucose plus SREBP-1c DN ($p < 0.05$, using paired t-test). DN; dominant negative.

5. Glucose-stimulation of GLUT2 gene expression

To observe the effect of glucose on GLUT2 and SREBP-1c mRNA and protein level, primary hepatocytes were maintained at the indicated concentration of glucose. As shown in Fig. 5A and B, GLUT2 mRNA and protein level increased with glucose concentration in a dose-dependent fashion. To confirm that SREBP-1c mediates glucose-stimulated GLUT2 expression, we transduced adenovirus containing SREBP-1c DN into the primary hepatocytes in the presence or absence of glucose. Real-time PCR (Fig. 5C, upper panel) and RT-PCR (Fig. 5C, lower panel) revealed that GLUT2 expression was increased by glucose (25 mM, $^{*}p < 0.005$, untreated vs glucose treated group) and the stimulatory effect was suppressed by SREBP-1c DN at 25 mM concentration of glucose ($^{\#}p < 0.05$, glucose treated vs glucose plus SREBP-1c DN). These data indicate that SREBP-1c is responsible for the glucose-stimulated GLUT2 gene expression.

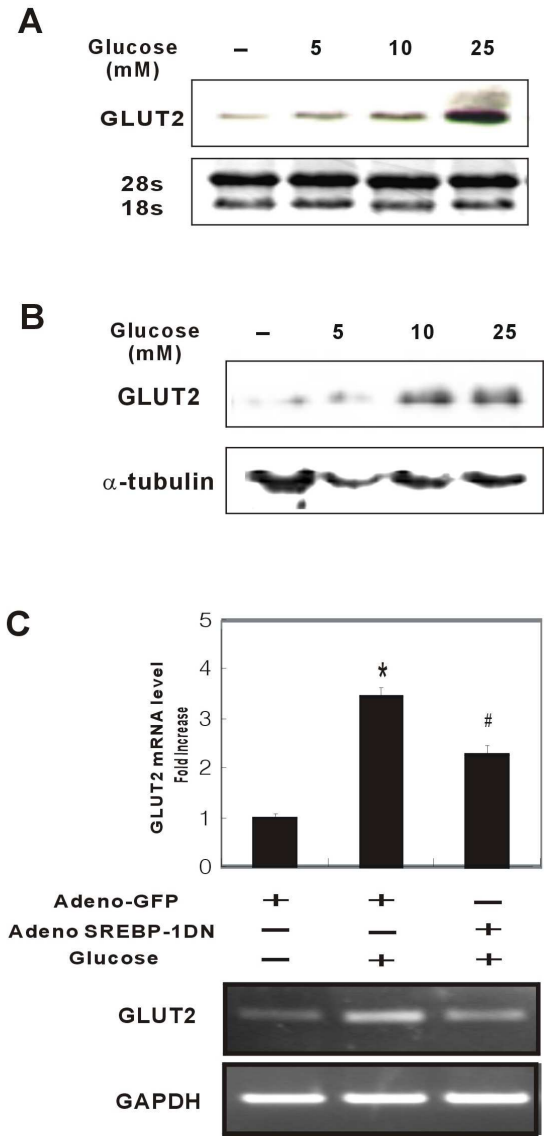

Figure 5. Glucose stimulation of GLUT2 gene expression. A and **B,** Primary hepatocytes, which were fasted for 24 h were maintained in media containing the indicated concentration of glucose for 16 h. GLUT2 mRNA and protein levels were quantified by northern (A) and western (B) blot analyses. The blots are representative of two different experiments. **C,** Effect of SREBP-1c DN on glucose stimulated

GLUT2 expression. Adenovirus containing SREBP-1c or null adenovirus (adeno-GFP) was transduced into primary hepatocytes at a titer of 5 plaque-forming units per cell for 2 h at 37℃. Cells were incubated in the presence (25 mM) or absence of glucose for 24 h, and RNA was harvested from the cells. The data were quantified as described in 'RESEARCH DESIGN AND METHODS'. The mRNA level was normalized to that of GAPDH. The results are the means ± S.D. of three independent experiments with triplicate measurements. *Adeno-GFP vs glucose ($p < 0.005$), $^{\#}$glucose vs glucose plus SREBP-1c DN ($p < 0.05$). GAPDH: glyceraldehydes 3-phosphate dehydrogenase.

6. Glucose increased the binding of SREBP-1 to the GLUT2 promoter in mouse primary cultured hepatocytes

To confirm the binding of SREBP-1 to the GLUT2 promoter by glucose in primary cultured hepatocytes, a ChIP assay was performed. Chromosomal DNA from primary cultured hepatocytes was cross-linked using formaldehyde, and an antibody specific to SREBP-1 was used to immunoprecipitate the chromatin fragment. A specific region of the GLUT2 promoter (-84/-76) was amplified by PCR. The binding of SREBP-1 to the GLUT2 promoter was increased by glucose or glucose/insulin treatment (Fig. 6A). The SREBP-1 binding to the GLUT2 promoter was increased in a dose-dependent manner (Fig. 6B). However, insulin did not affect the binding of SREBP-1 to the putative SRE. Since the chromatin immunoprecipation experiment is so critical to the conclusions, it would be helpful to show some further control for this experiment. To further confirm whether the insulin action worked well in cell lines, ChIP assay was performed using a known SRE site in a fatty acid synthase that responds directly to insulin. As shown Fig. 6C, insulin recruits SREBP-1c to FAS-SRE.

These results indicate that glucose could be one of the major factors in the binding of SREBP-1 to the GLUT2 promoter, which may help explain the upregulation of GLUT2 gene expression by glucose.

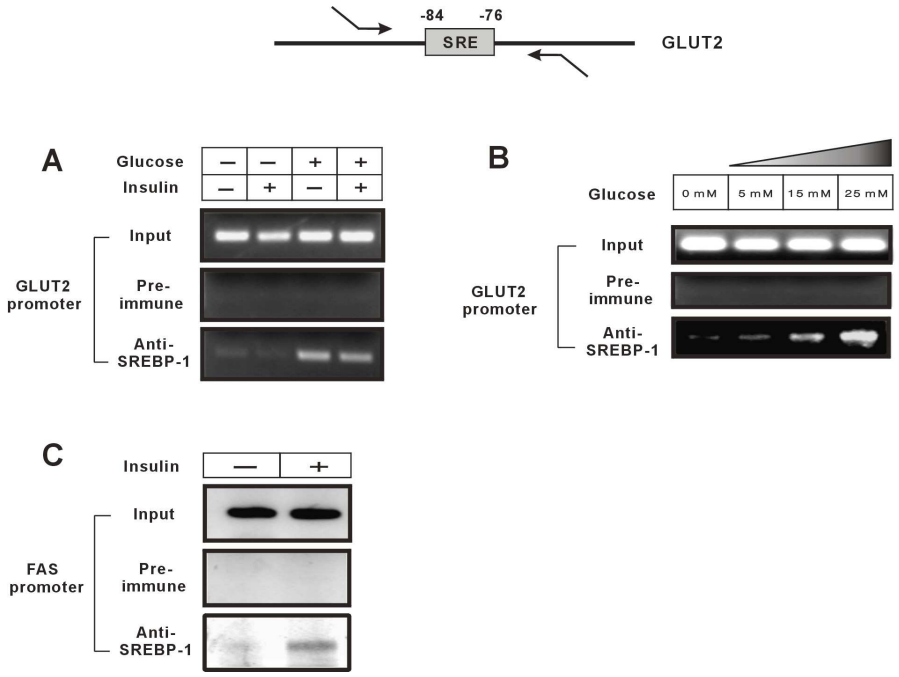

Figure 6. Chromatin immunoprecipitation (ChIP) assay. A, Effect of glucose or insulin on the SREBP-1 binding to putative GLUT2-SRE. Chromatin was precipitated using a SREBP-1 antibody from primary hepatocytes, and the GLUT2 promoter region was amplified by PCR. The amount of chromosomal DNA used in immuno-precipitation between groups was normalized by input chromatin (one hundredth of chromosomal DNA used for immunoprecipitation). **B,** Effect of glucose concentrations on the SREBP-1 binding to putative GLUT2-SRE. Glucose concentrations are shown on the top. **C,** Effect of insulin on the SREBP-1 binding to the FAS-SRE as a control. Detailed PCR conditions and methods of sample treatment are described in 'RESEARCH DESIGN AND METHODS.'

7. A model of GLUT2 gene regulation through glucose-stimulated SREBP-1c gene expression

Based on the results shown above, a possible model can be drawn (Fig. 7).

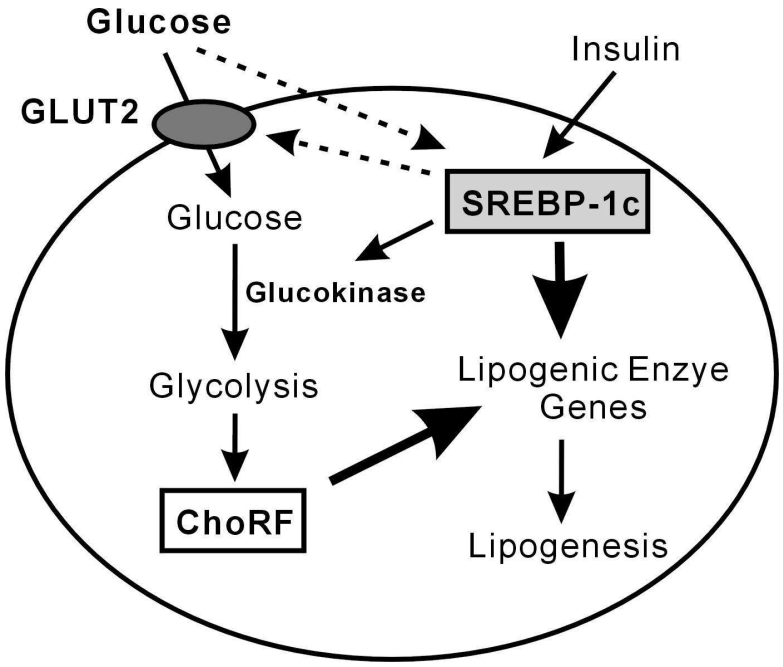

Figure 7. A model for the regulation of GLUT2 genes by glucose-stimulated SREBP-1c gene expression. The expression of lipogenesis related enzyme genes was known to be regulated by ChoRF and insulin-mediated SREBP-1c. But glucose was also possible to stimulate SREBP-1c gene expression and these upregulation of glucose-induced SREBP-1c increase the transcriptional level of GLUT2 gene in the hepatocytes.

Ⅳ. DISCUSSION

Recently, it has been suggested that the carbohydrate responsive element binding protein (ChREBP) plays a pivotal role in glucose regulated gene expression[36], whereas SREBP-1c acts as a major mediator of insulin action in the lipogenic genes or in hepatic glucokinase[37, 38] and lipogenic gene expression[9, 39]. However, glucose is required for lipogenic gene induction where insulin only plays a permissive role[15-17]. In insulin-resistant animals in which insulin signaling is impaired, SREBP-1c expression in liver was shown to be increased. For example, upregulation of SREBP-1c was reported in the leptin-deficient obese *ob/ob* mice, lipoatrophic mice overexpressing SREBP-1c in adipose tissue[19], insulin receptor substrate (IRS)-2 knockout mice[20], and rat primary hepatocytes infected with adenovirus-overexpressing dominant-negative Akt[12]. These observations suggest that there may be additional mechanism of SREBP-1c induction other than insulin signaling.

In this study, we demonstrated that glucose induced the gene expression of SREBP-1c and GLUT2 in a dose-dependent manner in the primary hepatocytes. Furthermore, expression of GLUT2 gene is decreased by adenoviral transduction of SREBP-1c DN in the mouse primary cultured hepatocytes, indicating that glucose stimulated GLUT2 expression is mediated by SREBP-1c. We have identified a functional SRE in the mouse GLUT2 promoter and demonstrated that SREBP-1c is the *trans*-acting factor mediating glucose-stimulated GLUT2 gene expression. In the

present study, the model of GLUT2 gene regulation through glucose-stimulated SREBP-1c gene expression was drawn in Fig. 7.

The question of how insulin induced SREBP-1c did not activate the GLUT2 gene expression, but glucose induced SREBP-1c increased GLUT2 mRNA level is not understood at the moment. As shown in Fig. 6A, SREBP-1c induced by insulin did not bind to GLUT2 promoter whereas SREBP-1c binding to GLUT2 promoter at high glucose concentration was increased. We speculate that this difference in the binding of SREBP-1c to SRE of GLUT2 promoter could be due to (i) difference in the coregulators recruiting SREBP-1c to SRE by insulin or glucose, or (ii) sequence specificity of GLUT2-SRE for SREBP-1c mediated by insulin or glucose signaling. Mostly, studies on the phenotypic changes in SREBP-1c knockout mice have been focused on the alteration in the lipid metabolism. On a normal diet, mRNAs encoding enzymes of fatty acid and triglyceride synthesis, including fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) are shown to be reduced in the liver of SREBP-1c deficient mice[3]. However, a compensatory increase in hepatic SREBP-2 mRNA, accompanied by increased mRNA level for cholesterol biosynthetic enzymes was observed[40]. Thus, these mice showed increase in the hepatic cholesterol content. The SREBP-1 deficient mice showed low level of plasma cholesterol and triglycerides[3]. At present, phenotypic changes in SREBP-1c knockout mice with regard to carbohydrate metabolism is not well studied. We assumed that in the insulin deficient or resistant state, where SREBP-1c transcription is low and gluconeogenesis is active in liver, the

glucose induced SREBP-1c may play a role in transporting glucose out of hepatocytes into blood, contributing to hyperglycemia.

Our studies may provide insight into the understanding of a role of GLUT2 on deranged carbohydrate metabolism in type 2 diabetes.

Ⅴ. CONCLUSION

1. Activation of SREBP-1c leads to the induction of hepatic mouse GLUT2 expression.

2. Glucose stimulated SREBP-1c expression induces the upregulation of the GLUT2 gene directly.

3. Steroid response elements (SRE) could be located between -84 and -76 on the GLUT2 promoter.

4. In the insulin-deficient or –resistance state, where gluconeogenesis is active in liver, the glucose-induced SREBP-1c may play a role in transporting glucose out of hepatocytes into blood, contributing to hyperglycemia.

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ABSTRACT (IN KOREAN)

포도당에 의해 SREBP-1c를 통한 간장형 포도당 운반체의 전사 조절

<지도교수 **안용호**>

연세대학교 대학원 의과학과

임 승 순

간장에서 GLUT2 유전자의 발현은 혈당이 높으면서 인슐린이 많이 분비되는 음식물 섭취 후 상태에서 증가되지만, 인슐린이 부족하면서 혈당이 높은 제2형 당뇨병에서도 유전자발현이 증가된다. 이러한 현상은 포도당과 인슐린이 GLUT2 유전자 발현에 서로 다르게 작용한다는 사실을 의미한다. 유전자 전사조절에서 포도당과 인슐린의 협력적인 관계는 스테롤 조절부위 결합 단백질 (SREBP-1c)을 통하여 이루어진다. 하지만, 이런 이론으로는 인슐린이 분비되는 정상 상태의 GLUT2 발현조절은 설명이 가능하지만, 포도당 농도는 높지만 인슐린 농도가 낮거나, 혹은 인슐린 저항성이 있는 제2형 당뇨병에서는 GLUT2 발현이 증가되는 이유를 설명할 수 없게 된다. 따라서 본 연구에서는 GLUT2 발현이 glucose-insulin-SREBP-1 axis에 따라 조절되는 것이 아니라 인슐린과는 독립적으로 (인슐린 비의존적) 포도당에 의해 활성조절 된다는 사실을 확인하고, 이 과정에서 SREBP-1c가 직

접 관여하며, GLUT2 프로모터 내에 SREBP-1c가 결합하는 *cis*-element (SRE) 부위를 규명하고자 하였다.

마우스 primary hepatocytes를 분리하여 인슐린과 포도당에 의한 SREBP-1c와 GLUT2의 발현을 northern blot과 Real-time PCR로 측정한 결과 SREBP-1c가 포도당에 의해서도 발현이 증가하는 것을 확인하였다. 간장 세포주에서 SREBP-1c를 과발현시킨 후 GLUT2 프로모터의 활성도를 측정한 결과 SREBP-1c에 의해 GLUT2의 활성이 증가였다. GLUT2 프로모터 내에 SREBP-1c가 결합하는 부위를 찾기 위해 순차적으로 제거한 후 GLUT2의 프로모터 활성을 확인한 결과 -166과 -57사이에 존재함을 알게 되었다. SREBP-1c 아데노 바이러스를 이용하여 마우스 primary hepatocytes에서 endogenous GLUT2의 발현을 확인하였으며 SRE의 보존 유전자 염기서열을 이용하여 -84~-76bp 부위에 SRE가 존재함을 유추하게 되었다. 재조합 SREBP-1c 단백질을 이용하여 결합여부를 확인한 결과 이 부위에 SREBP-1c가 잘 결합하는 것을 확인하였고, 크로마틴 항체침전법을 사용하여 *in vitro*에서 SREBP-1c가 포도당에 의해 GLUT2 유전자의 프로모터 부위에 직접 결합하여 발현을 조절한다는 사실을 확인하였다.

핵심 되는 말 : 간장, 간장형 포도당운반체 (GLUT2), 포도당, 스테롤 조절부위 결합단백질 (SREBP-1c)