Regulation of GLUT2 Glucose Transporter Gene Promoter Activity by Peroxisome Proliferator-Activated Receptor ɣ in Hepatocytes

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Directed by Professor Yong-Ho Ahn

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Seung-Soon Im

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This certifies that the master's thesis of
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June 2002

감사의 글

너무나도 부족한 저에게 이런 논문을 쓸 수 있도록 허락하시고 은혜와 지혜를 충만하게 베풀어주신 우리 주 하나님께 먼저 감사와 영광을 돌립니다. 이 논문이 있기까지 세심한 배려와 아낌없는 격려로 지도를 베풀어주신 은사 안용호 선생님께 진심으로 머리 숙여 감사를 드립니다. 또한 부족한 논문을 심사하여 주신 김경섭 선생님과 차봉수 선생님께 깊은 감사를 드립니다. 그리고 언제나 따뜻한 관심과 도움을 주신 오상환 선생님, 허만욱 선생님, 김건홍 선생님께도 감사를 드립니다. 무엇보다도 이 논문의 모든 실험을 함에 있어서 많은 도움을 주시고 조언을 아끼지 아니하시며 관심과 열의로 지도해주신 김재우 선생님께 특별히 감사를 드립니다.

아울러 본 연구기간 중에 도움을 주신 차지영 선생님, 김소연 선생님, 이천수 선생님, 임미숙 선생님에게 감사드리며, 어려움 중에 함께 했던 모든 생화학-분자생물학 교실 선생님들에게 고마운 마음을 전합니다. 또한 관심과 사랑으로 기도해 주셨던 모든 분들에게 감사를 드립니다.

마지막으로, 언제나 부족한 저를 위해 헌신적인 기도와 사랑으로 보살펴 주신 부모님과 가족들 그리고 항상 기도 가운데 용기와 격려를 아끼지 않는 사랑하는 상희씨에게 감사의 마음과 함께 이 논문을 바칩니다.

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Regulation of GLUT2 Glucose Transporter Gene Promoter Activity by Peroxisome Proliferator-Activated Receptor γ in Hepatocytes

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GLUT2 glucose transporter is mainly expressed in hepatocytes and pancreatic β-cells and plays a critical role in glucose homeostasis in living organism. The expression of GLUT2 is reported to be regulated by several liver-specific transcription factors, and there are evidences that peroxisome proliferator-activated receptor γ (PPARγ) may participate in regulating the expression of genes involved in glucose metabolism in liver. To this end, we examined human, rat, and mouse promoter activities exerted by PPARγ, and observed that mouse GLUT2 promoter was the most activated in primary mouse hepatocytes as well as various hepatic cell lines by troglitazone treatment. Serial deletion study of the construct revealed that the promoter activity was

dramatically decreased when -166 region was deleted. DNase I footprinting assay using liver nuclear extract clearly showed the protection between -206 and -147, suggesting the presence of PPAR response element (PPRE) in this region. The protein binding to this site was further investigated by electrophoretic mobility shift assay (EMSA) with a combination of competition and supershift assay. From this experiment, we have localized the PPRE in the region of -197/-184.

Introduction of a mutation in this region resulted in the dramatic decrease in the promoter activity. Also, PPARγ increased the endogenous GLUT2 mRNA expression in primary mouse hepatocytes. From these results, it is concluded that the mouse GLUT2 promoter can be regulated by PPARγ, which may explain a role of liver in the regulation of blood glucose level when troglitazone was administered to type 2 diabetic patients.

Key Words : GLUT2, PPARγ, troglitazone, liver, hepatocyte

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I. Introduction

The GLUT2, a specific facilitative glucose transporter, is mainly expressed in hepatocytes[1] and pancreatic β-cells,[2] and has a higher Km value compared to other types. The glucose metabolism in liver begins or ends with the movement of glucose into or out of the hepatocyte through this type of glucose transporter.[3] Several liver specific transcription factors, such as hepatocyte nuclear factor-1 (HNF-1), HNF-3 and CCAAT/enhancer binding protein (C/EBP), known to be able to regulate the GLUT2 promoter activity.[4,5] It is believed that these transcription factors play important roles for GLUT2 expression during differentiation of hepatocyte as well as glucose homeostasis.

The principal pathology in the type 2 diabetes may be insulin resistance[6,7] and impaired glucose tolerance which may be caused by impaired glucose transport into muscle or fat, and inappropriate glucose disposal in the liver.[8] Recently, peroxisome proliferator-activated receptor γ (PPARγ) agonists, thiazolidinediones (TZDs) have been drawn much attentions in the treatment of type 2 diabetes. These drugs act as ligands for the nuclear receptor PPARγ and enhance the insulin sensitivity of skeletal muscle,[9] adipose tissue,[10,11] liver,[12] and pancreatic β-cells.[13] In addition to their effects on blood glucose regulation, they have been known to improve the syndromes associated with insulin resistance.[14,15] Currently, studies on the mechanisms how glucose is disposed in the muscle and adipose cells[16] have been reported by many investigators, mostly emphasizing in terms of GLUT4 translocation or transcriptional control in those tissues.[17,18,19] However, in spite of its important role in the blood glucose regulation, the effect of TZDs on the liver is not well understood to date.

There are growing evidences that PPARγ may participate for regulating the expression of genes involved in glucose metabolism in liver. Although the role of PPARγ in liver has been underestimated because the expression level of PPARγ in liver is lower than adipose tissue, it was reported that obesity and nutrition can upregulate PPARγ expression in liver.[20] Moreover, PPARγ

agonists treatment caused the increased glucose uptake in primary hepatocytes[21]. These suggest that PPARγ is important for the glucose metabolism in the alteration of physiologic or disease status. Therefore, GLUT2, which is responsible both for hepatic glucose entry and output, could be a target of TZDs action if the drugs sensitize the liver to control blood glucose level.

In this study, we investigated GLUT2 promoter activity in response to PPARγ and TZDs, and we were able to localize the PPRE in the -197/-184 region of mouse GLUT2 promoter. The binding of PPARγ was confirmed by DNase I footprinting assay and electrophoretic mobility shift assay (EMSA). We also showed that PPARγ expression could increase the endogenous GLUT2 mRNA expression in primary mouse hepatocytes, suggesting that PPARγ plays an important role in the regulation of blood glucose level by the liver.

Ⅱ. Materials and Methods

1. Materials

Troglitazone was a gift from Sankyo Co. Ltd. (Tokyo, Japan). 9-*cis* retinoic acid was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Troglitazone concentration was adjusted to 10 mM in 19% (w/v) BSA and 5% (v/v) DMSO.

2. Construction of the mouse GLUT2 promoter

The 5' flanking region of mouse GLUT2 promoter was obtained from the mouse genomic DNA by polymerase chain reaction (PCR) and inserted into the pCRII-TOPO vector (Life Technologies, Gaithersburg, MD, USA). The KpnI/XhoI fragment of pCR-mGT2-1112 subcloned into the KpnI/XhoI site of the pGL3B vector (Promega, Madison, WI, USA). Expression plasmids, pCMX-mPPARγ, pCMX-mRXRα were kind gifts from Drs, R. M. Evans and D. J. Mangelsdorf.[22,23] Control vector, pCMX was prepared from pCMX-mRXRγ by excising out the 1.5 kb fragment of mRXRγ cDNA.

Promoter constructs, M-p (mouse ; -1112/+1), M-d (mouse ; -486/+1), H-p (human ; -1125/+1), R-p (rat ; -910/+1), R-d (rat ; -731/+1) were amplified by

PCR using the following primers respectively : (sense, 5'-CCCAG TTTCC TTCCG ACCTT-3', antisense, 5'-TTGTG TGTGT GTGGA ATTGT-3'), (sense, 5'-CACCT TGCAG AGGTG ACAGA-3', antisense, 5'-TTGTA CTAGT TGGGA GTCCT-3'), (sense, 5'-ATGGA TGAGG AAAGG TGGGA-3', antisense, 5'-TGTTG CGTGC GGAGT TGTTG-3'). Deletion mutants of mouse GLUT2 promoter were made from pMGT2-1112. pMGT2-890 was prepared by excising out 222 bp fragment of SalI and EcoRI double digestion. pMGT2-389 was constructed by digesting with SalI and PstI from the pMGT2-1112. Deletion constructs of mouse GLUT2 promoter were made by digestion of pMGT2-1112 after EcoRI sites generated by PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), and named pMGT2-283, pMGT2-166, and pMGT2- 57, respectively. The PPRE truncated construct, pMGT2d-283/-166, was also made from pMGT2-389 with the same method described above. Mutant constructs pMGT2m-203 (M1), pMGT2m-196 (M2), pMGT2m-189 (M3) and pMGT2dm-196/189 (M2+3) were produced by introducing substitution mutations into pMGT2-389 using site-directed mutagenesis. The sequences of constructs were confirmed by DNA sequencing.

3. Primary hepatocytes preparation from mouse liver

Primary hepatocytes were isolated from ICR mouse (approx. 30 g) by the collagenase perfusion method.[24] Livers of 7-week-old mice were perfused through the portal vein with a perfusion solution I [10 mM N-(2-hydroxy-ethyl)piperazine-N'-(2-ethane-sulfonic acid) (HEPES), pH 7.4, 142 mM NaCl, 6.7 mM KCl, and 2.5 mM ethyleneglycol-bis-(2-aminoethylether)-N,N,N',N'-tetra-acetic acid (EGTA) (Sigma-Aldrich Co., St. Louis, MO, USA)]. All the solutions were pre-warmed at 37℃ in water bath. The perfusion solution I was first passed more than 400 ㎖ through the liver for 15～20 min and the perfusion solution II [4.8 mM $CaCl_2$ and 0.5 ㎎/㎖ collagenase A (Roche Diagnostics GmbH, Mannheim, Germany)] was circulated for 15～20 min. After purfusion, liver was rapidly removed and appropriate amount of cold plating media was poured into petri dish and hepatocytes were dissociated by gentle shaking. Cells were collected by filtering through the mesh. The Dulbecco's modified Eagles' medium (DMEM) (Life Technologies) containing 25 mM glucose, 100 unit/㎖ penicillin, 100 ㎍/㎖ streptomycin, 10% (v/v) heat inactivated fetal calf serum (FCS) (Life Technologies) were added to bring the volume to 50 ㎖. The cells were gently resuspended by pipetting 4～5 times. The isolated hepatocytes were then washed for 2 min at 50 ×g at 4℃ with DMEM and then suspended in a small volume of DMEM containing 25 mM

glucose, 10 nM dexamethasone (Sigma-Aldrich Co.) and 0.1 unit/ml insulin (Sigma-Aldrich Co.).

4. Transient transfection

Alexander cell line was purchased from the ATCC and maintained in modified Eagles' medium (MEM), supplement with 10% (v/v) FCS, 100 unit/㎖ penicillin, 100 ㎍/㎖ streptomycin, and 5 × tylosine tartrat solution (SERVA electrophoresis GmbH, Heidelberg, German). Cells used in this experiment were maintained as monolayer cultures and grown in appropriate media. Plasmid DNAs were purified using Qiagen Midiprep kit columns (QIAGEN, Valencia, CA, USA). Cells were plated in six-well tissue culture plates at a density of 1×10^6 cells/well in 2 ㎖ of medium. After a 20-h attachment period, transfections were performed with LipofectAMINE PLUS reagent (Life Technologies), according to the manufacturer's protocol. Briefly, 0.5 ㎍ of each construct of GLUT2 promoter, 0.1 ㎍ of pCMV-β-galactosidase and 4 ㎕ of plus reagent and 2 ㎖ of lipofectamine in 200 ㎖ of OPTI-MEM I (Life Technologies) media lacking serum were mixed and added to cells. After 3-h, the medium containing the LipofectAMINE-DNA complex was removed and replaced by appropriate media (containing serum and antibiotics). After 24-h transfection, the medium containing 10 mM of troglitazone and 2 mM of 9-*cis*

retinoic acid (Sigma-Aldrich Co.) was treated into cells. Cells were then cultured further for 24-h and harvested in reporter lysis buffer (Promega, Madison, WI, USA). The lysed cells were centrifuged to remove cell debris completely and the supernatant was collected. Luciferase assays were conducted with 10 ㎕ of cell extracts and 50 ㎕ of luciferase assay reagent (Promega). β-galactosidase activity was determined with 10 ㎕ of cell extract and 190 ㎕ of pyranoside in as colorimetric assay. Luciferase data were expressed as luciferase activity corrected by β-galactosidase activity in the cell lysate. Each transfection was performed in triplicate and repeated three to five times.

5. Nuclear extracts preparation

Nuclear extracts from liver of male Sprague-Dawley rats and ICR mouse were prepared as described by Gorski's protocol.[25] Briefly, livers were minced and homogenized using Polytron (OMNI International, Waterbury, CT, U.S.A.) in 80 ㎖ of homogenation buffer [10 mM HEPES, pH 7.6, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 M sucrose, 10% (v/v) glycerol, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 ㎍/㎖ proteinase inhibitor cocktail (pepstatin A, leupeptin, chymostatin, antipain), 1% (w/v) low fat milk]. These homogenates were subjected to sucrose gradient ultracentrifuge at 24,000

rpm for 1-h. Nuclear pellets were resuspended in 2 ㎖ of nuclear digestion buffer [10 mM HEPES, pH 7.6, 100 mM KCl, 0.1 mM EDTA, 10% (v/v) glycerol, 3 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 5 ㎍/㎖ proteinase inhibitor cocktail], and 1/10 volume of 4 M ammonium sulfate, pH 7.9 was added. Following centrifuge at 35,000 rpm for 1-h, supernatant was transferred and ammonium sulfate (0.3 g/㎖) was added. After centrifugation at 35,000 rpm for 20 min, the pellet was dissolved in 1 ㎖ of dialysis buffer [25 mM HEPES, pH 7.6, 0.1 mM EDTA, 40 mM KCl, 10% (v/v) glycerol, 1 mM DTT] and subjected to dialysis for 4-h. The insoluble fractions were removed by centrifuge, and clear nuclear extracts were stored at -70℃. Protein concentration was determined by the method of Bradford.[26]

6. DNase I footprinting assay

DNA fragments covering from -389 to +1 of coding strand of mouse GLUT2 gene were labeled in one strand and purified as follows. The promoter region between -389 to +1 was removed from pMGLUT2 by digestion of KpnI and XhoI, and subcloned into the KpnI and XhoI site of pBluescript II SK(+) vector. The plasmids were digested by EcoRI to obtain 5' overhanging ends and 3' overhanging ends and labeled using Klenow fragment and [α-^{32}P] dATP. Labeled DNA was purified from 5% (w/v) polyacrylamide gel by crush

method. DNA-protein binding reactions were performed using 50,000 cpm (approx. 1 ng) of probe per reaction in a solution containing 10 mM HEPES, pH 7.9, 60 mM KCl, 7% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, 2 ㎍ of poly(dI-dC) (Amersham Pharmacia Biotech., Bucks., U.K.), and the indicated amount of nuclear extracts. After 20 min of incubation on ice, 5 ㎕ of DNase I freshly diluted in a solution containing 10 mM HEPES, pH 7.9, 60 mM KCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$, and 7% (v/v) glycerol was added to the reaction and kept at room temperature for 2 min. Dilution folds of DNase I were ranged from 1:200 to 1:400 of stock (10 units/㎕) depending on the amount of protein present in the reaction. Digestion reactions were stopped by adding 80 ㎕ of a stop solution containing 20 mM Tris-Cl, pH 8.0, 20 mM EDTA, 250 mM NaCl, 0.5% (w/v) sodium dodecyl sulfate (SDS), 4 ㎍ of yeast tRNA, and 10 ㎍ of proteinase K. The samples were incubated for 30 min at 45℃, extracted with phenol/chloroform, ethanol-precipitated, and resuspended in formamide dye. The samples were resolved in 6% (w/v) polyacrylamide/7 M urea sequencing gel. The dried gels were exposed to X-ray film at -70℃ with an intensifying screen. The protected regions were mapped with reference to the migration of Maxam-Gilbert A+G sequencing products.

7. Electrophoretic mobility shift assay (EMSA)

Ten pmoles of single stranded sense oligonucleotide were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (TaKaRa, Shiga, Japan) and annealed with 50 pmoles of unlabeled antisense oligonucleotides. The resulting double stranded oligonucleotides were purified by Sephadex G50 (Amersham Pharmacia Biotech.) spin column. 50,000 cpm (approx. 0.003 pmoles) of probe were incubated with 5 ㎍ of nuclear extract from mouse or rat liver for 10 min on ice. The binding reactions[27] were carried out in 20 ㎕ of binding buffer containing 10 mM HEPES, pH 7.9, 60 mM KCl, 1 mM DTT, and 1 ㎕ of poly(dI-dC). To perform supershift assay, the binding mixtures were incubated for 10 min at room temperature in the presence of 1 ㎕ of antibodies. Protein-DNA complexes were resolved from the free probe by electrophoresis at 4℃ on a 4% (w/v) polyacrylamide gel in 0.25 × TBE buffer (1 × TBE contained 9 mM Tris, 90 mM boric acid, 20 mM EDTA, pH 8.0). The dried gels were exposed to X-ray film at -70℃ with an intensifying screen. The oligonucleotides used in EMSA were shown in Table 1.

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

Total RNA was isolated from primary hepatocytes using TRIzol® reagent by manufacturer's protocol (Life Technologies). For RT-PCR, first strand cDNA was synthesized from 3 ㎍ of total RNA using random nanomer and Superscript II reverse transcriptase (Life Technologies). One ㎕ of the reverse transcription reaction mixture was amplified with primers specific for GLUT2, PPARγ, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) in the total volume of 50 ㎕. Linearity of the PCR was tested by amplification cycles between 25～30. According to test amplification profile, samples were amplified for 28 cycles using the following parameters: 94℃ for 30 sec, 58℃ for 30 sec, 72℃ for 30 sec. G3PDH was used as an internal control for quality and quantity of RNA. The PCR products were subjected to electrophoresis on 1% (w/v) agarose gel and the quantities of PCR products were analyzed by Molecular Analyst II (Bio-Rad, Hercules, CA, USA). Primers used in PCR were summarized in Table 1.

9. Statistics

All transfection studies were carried out in three separate experiments, where triplicate dishes were transfected. Data are expressed as means ± S.D. and compared by the Student's t-test.

Table 1. The sequences of the oligonucleotides used in this experiment.

Oligonucleotide	Sequence
GLUT2-Ex2-sense	5'-ATCACCGGAACCTTGGCTTCACT
GLUT2-Ex5-sense	5'-GGCTAATTTCAGGACTGGTT
GLUT2-Ex6R-antisense	5'-TTTCTTTGCCCTGACTTCCT
PPARγ-sense	5'-GGATCCAACATGGTGGACACAGA
PPARγ-antisense	5'-GTCGACGCCATCTCAGGAAAGATC
G3PDH-sense	5'-AACCACAGTCCATGCCATCAC
G3PDH-antisense	5'-TCCACCACCCTGTTGCTGTA
GT2-PPRE	5'-CTGTGCTCAAGCCAC**AAGTCATTGGGGT**AAAGGGT
M1	5'-CTGTGCTC**GAATTC**CAAGTCATTGGGGTAAAGGGT
M2	5'-CTGTGCTCAAGCCAC**GAATTC**TTGGGGTAAAGGGT
M3	5'-CTGTGCTCAAGCCACAAGTCAT**GAATTC**AAAGGGT
M2+3	5'-CTGTGCTCAAGCCAC**GAATTC**T**GAATTC**AAAGGGT

Mutated regions are marked by bold letters and putative PPRE sequence was underlined.

Ⅲ. Results

1. Cloning of mouse GLUT2 promoter and comparison of homology between species

As previously reported, the structure of the rat GLUT2 gene has somewhat different features compared to those of human and mouse (Fig. 1),[28,29] thus we first compared sequences between human, rat, and mouse GLUT2 promoter regions. In order to compare upstream regions between the species in detail, we cloned 1.5 kb mouse gene upstream of known promoter by polymerase chain reaction based on the homology between rat and mouse gene. Human GLUT2 gene sequence was obtained from human genome project. As shown in Fig. 1, the 5'-flanking sequence just upstream of ATG codon showed great similarity between species, however, the promoter regions harboring exon 1a of rat gene did not show significant similarities. Thus, we considered the possibility of at least two alternative promoter usage of GLUT2 gene and designated them as distal and proximal promoters. Based on this homology, we constructed promoter-luciferase constructs and used them for experiment including transient transfection study (Fig. 2A). All the nucleotide numbers of constructs were counted from +1 of ATG codon.

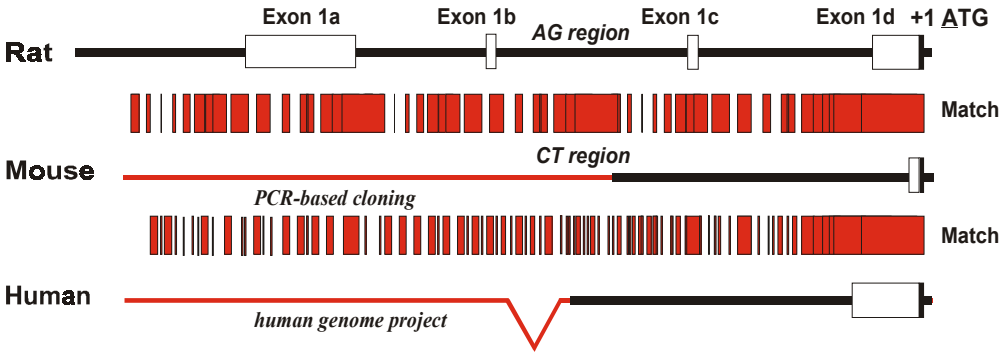
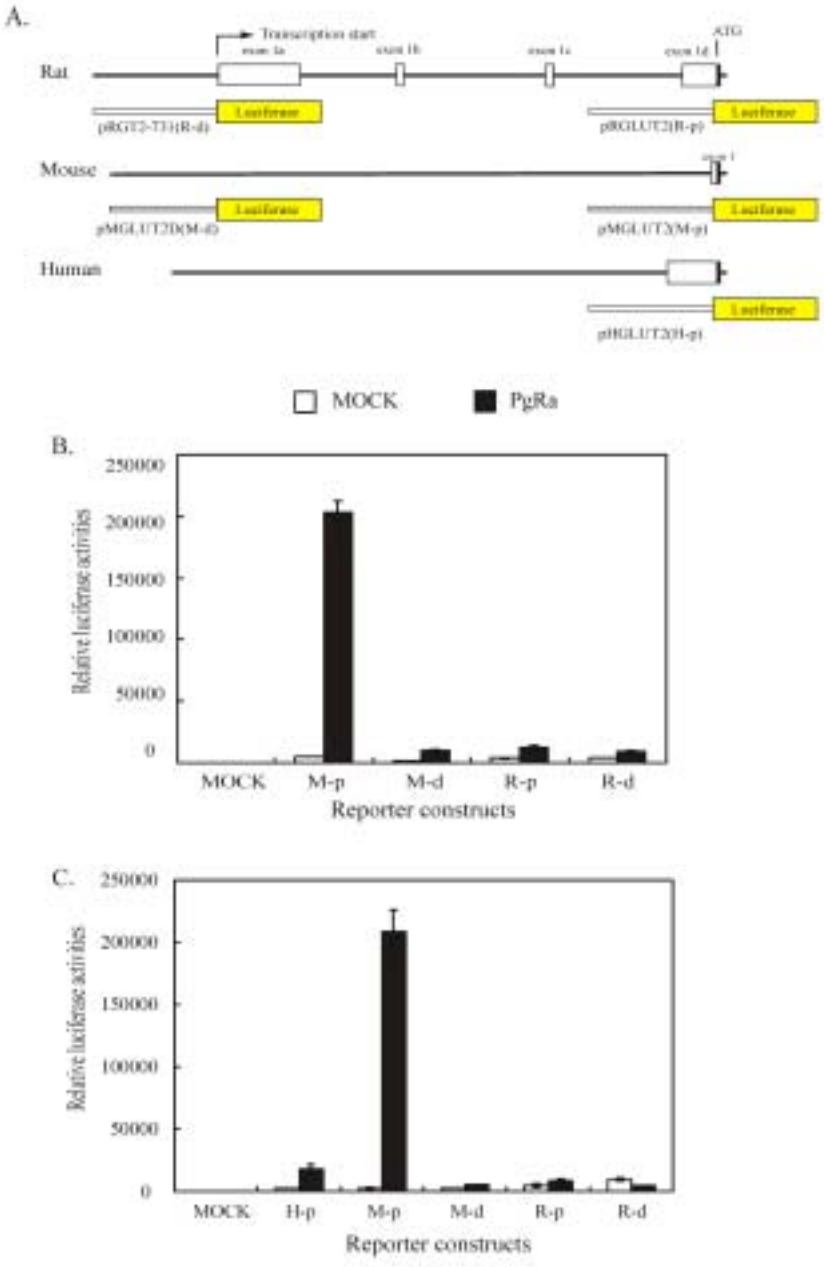


Fig. 1. The comparison of promoter sequence between species. Computer based sequence homology was examined between rat, mouse, and human GLUT2 promoter region. Previously reported exon structure was showed by open boxes, and coding sequence including ATG codon was marked. Rat and mouse genome have long repeats of AG or CT respectively, whereas human promoter does not contain the repeats. The nucleotide similarity between three species was showed by filled boxes.

2. PPARγ activates mouse GLUT2 promoter in various cell lines

To determine whether PPARγ can regulate GLUT2 promoter activity, we constructed GLUT2 promoter from each species into pGL3-basic vector and tested the responsiveness to PPARγ in various cell lines. Because pancreatic β-cell specific PPRE was reported to be localized in the distal promoter of rat GLUT2,[30] we included the potential promoters either distal or proximal region for the experiment. The reporter vectors, pRat GLUT2-distal/proximal, pMouse GLUT2-distal/proximal and pHuman GLUT2-proximal (Fig. 2A), were transfected into cell lines such as fibroblast cell line (CV-1), hepatic cell lines (Alexander and HepG2), and beta cell line (HIT-T15). As shown in Fig. 2, the mouse GLUT2 promoter containing -1112 to +1 region was activated over 40 folds by PPARγ compared to control null reporter in Alexander and HepG2 cells (Fig. 2B and 2C). This transactivation was also observed when other cell lines were used, although the fold increases were varied according to the cell lines (Fig. 2D and 2E). These results suggested that PPARγ can act as a transactivator in the regulation of the mouse GLUT2 promoter in liver cells. On this basis we have searched for the presence of PPRE in the promoter region of the mouse GLUT2 gene.


A.
Transcription start
ATG
Rat
Luciferase
Mouse
Human
MOCK
PgRa
B.
Relative luciferase activities
250000
200000
150000
100000
50000
0
MOCK
M-p
M-d
R-p
R-d
Reporter constructs
C.
Relative luciferase activities
250000
200000
150000
100000
50000
0
MOCK
H-p
M-p
M-d
R-p
R-d
Reporter constructs

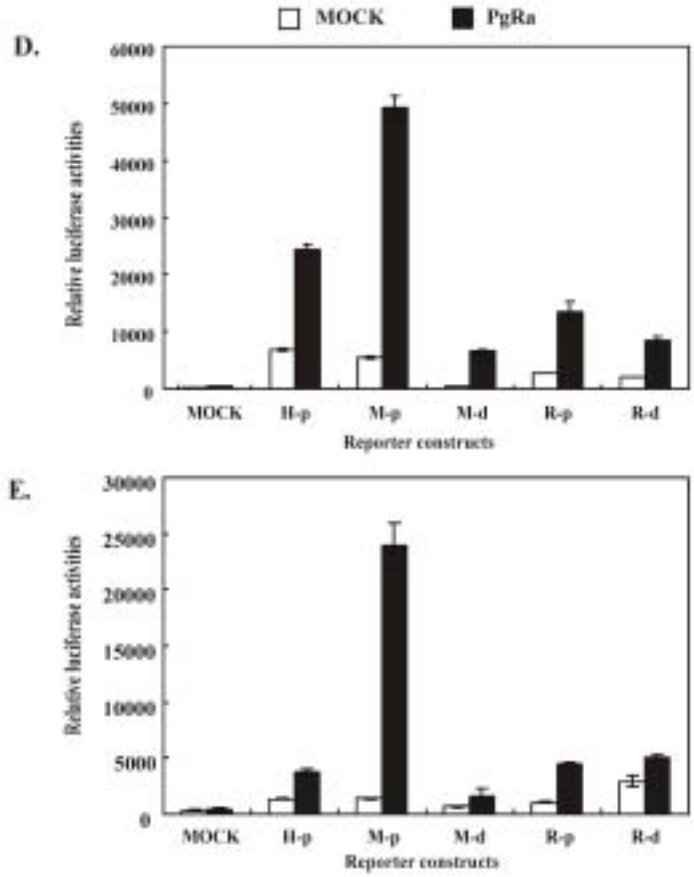


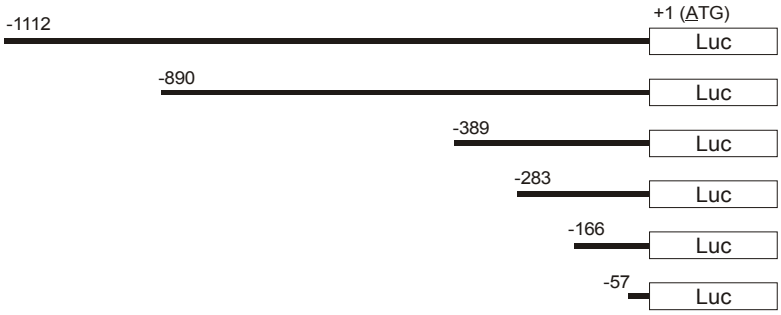
Fig. 2. The transient transfection study using rat, mouse, and human GLUT2 promoter in various cell lines. Luciferase reporter under the control of GLUT2 promoters was cotransfected into each cell lines with expression vectors of PPARγ and RXRα. Appropriate ligands for receptors were treated after transfection; 20 μM of troglitazone for PPARγ and 1 μM of 9-*cis* retinoic acid for RXRα were used. A. Constructs of GLUT2 promoter in pGL3 basic cloning vector containing proximal or distal region. B. Effect of PPARγ overexpression on the GLUT2 promoter activities in Alexander cells. Cells were cotransfected with each luciferase construct with or without PPARγ/RXRα complexes as indicated. All results were normalized with respect to β-galactosidase activities. The results were shown as relative luciferase activities compared to that of pGL3-basic without PPARγ. C. HepG2 cells. D. CV-1 cells. E. HIT-T15 cells. Normalized luciferase activities are shown as means ± S.D. of three independent experiments in a triplicate.

3. A PPRE is located on mouse GLUT2 promoter

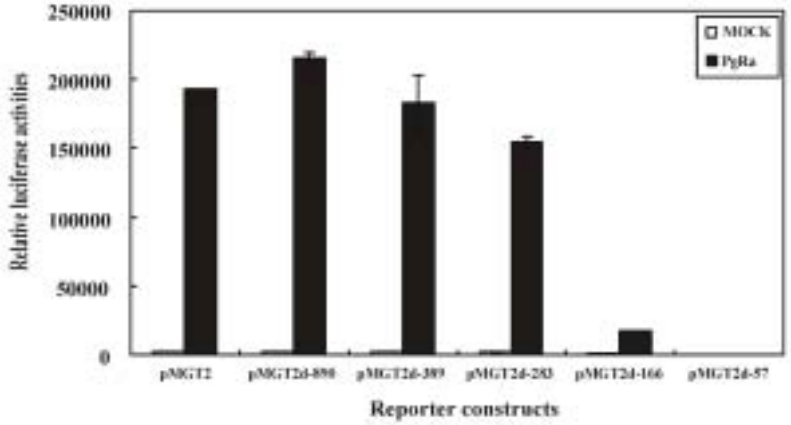
To identify the functional PPRE regions involved in regulating the mouse GLUT2 promoter activity, serially deleted 5' flanking regions (from -1112, -890, 389, -283, -166, and -57 to +1, shown in Fig. 3A) were cloned into the reporter plasmid pGL3 basic. To tested their responsiveness to PPARγ, reporter constructs were transfected into Alexander cells. As shown in Fig. 3B, pMGT2-1112, pMGT2-890, pMGT2-389, and pMGT2-283 were remarkably activated by coexpression of PPARγ and RXRα in the presence of troglitazone and 9-cis retinoic acid. However, deletion down to -166 (pMGT2-166) and -57 (pMGT2-57) resulted in loss of promoter activation. Further decrease of promoter activity in pMGT2-57 was thought to be a result from removing the binding site of basal transcription machinery. Same result was obtained with mouse primary hepatocytes culture (Fig. 3C). When -283 to -166 was truncated from the promoter, the promoter activity was dramatically decreased (Fig. 3D). These results suggested that PPARγ, heterodimerized with RXRα, activated the mouse GLUT2 in liver cells and the activation required the sequences between -283 and -166 of GLUT2 promoter, which may contain the putative PPRE. DNase I footprinting assay using liver nuclear extract revealed three protein binding sites around this region (Fig. 4). Of these, the distinct DNA sequence elements called DR+1 sites was found on

the protected region between -206 and -147, suggesting the presence of putative PPRE in this region which mediates the PPARγ regulated transcription of mouse GLUT2 promoter.

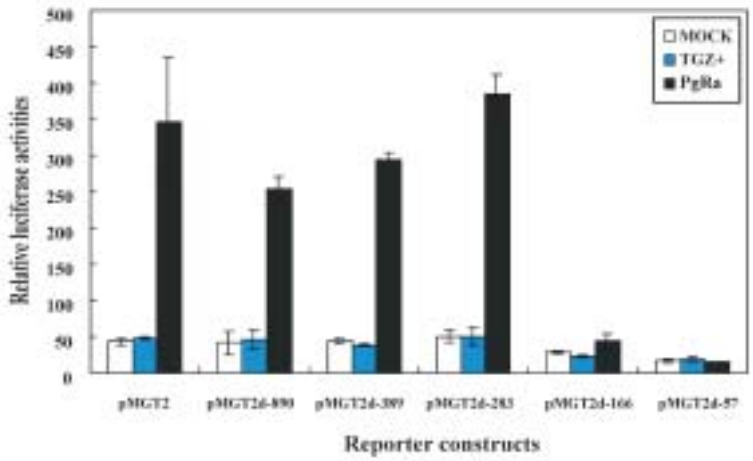
A.


+1 (ATG)
-1112
Luc
-890
Luc
-389
Luc
-283
Luc
-166
Luc
-57
Luc

B.


MOCK
PgRa
Relative luciferase activities
250000
200000
150000
100000
50000
0
pMGT2
pMGT2d-890
pMGT2d-389
pMGT2d-283
pMGT2d-166
pMGT2d-57
Reporter constructs

C.


MOCK
TGZ+
PgRa
Relative luciferase activities
500
450
400
350
300
250
200
150
100
50
0
pMGT2
pMGT2d-890
pMGT2d-389
pMGT2d-283
pMGT2d-166
pMGT2d-57
Reporter constructs

D.

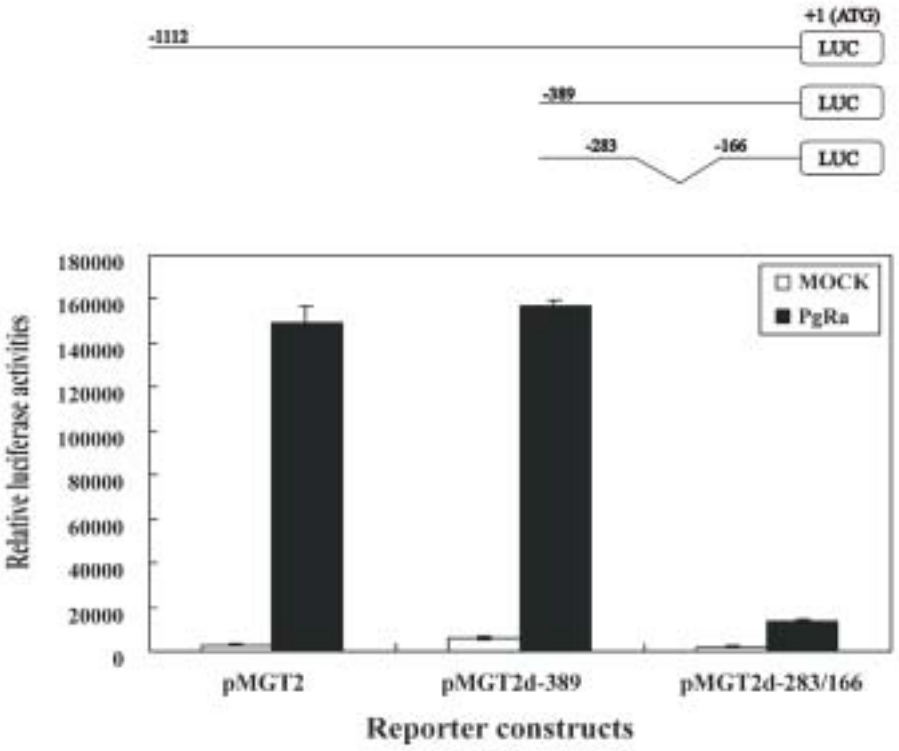


Fig 3. Localization of PPRE in mouse GLUT2 promoter. A. To localize the PPRE on the mouse GLUT2 promoter, serial deletion constructs were made. The indicated numbers represent bases upstream of ATG codon. B. Transfection study in Alexander cell line. Cells were cotransfected with each luciferase construct with or without PPARγ /RXRα complexes as indicated. All results were normalized with respect to β-galacto-sidase activities. The results were shown as relative luciferase activities. C. Transfection study in mouse primary hepatocyte culture. D. Truncation of the region from -283 to -166 in pMGT2d-389 vector resulted in the loss of responsiveness to PPARγ in Alexander cells. Normalized luciferase activities are shown as means ± S.D. of three independent experiments in a triplicate.

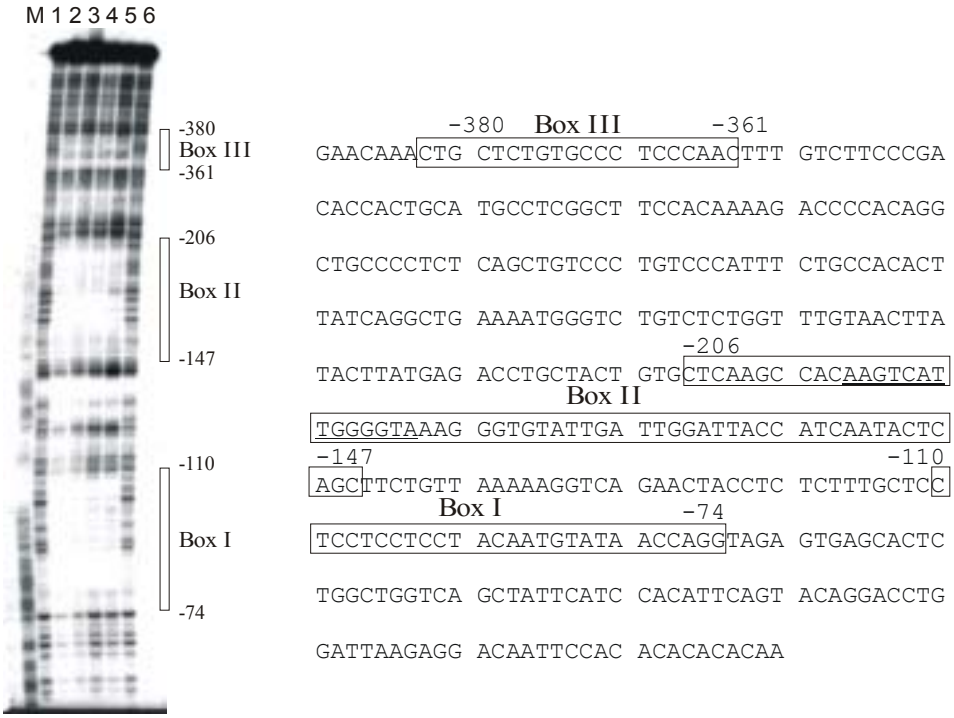


Fig 4. DNase I footprinting assay of the mouse GLUT2 promoter region. DNase I footprinting assay was performed as described in the Materials and Methods section using rat liver nuclear extract. The probe was [^{32}P]-end labelled fragment of -389 to +1. M, Maxam-Gillbert A+G tract; lane 1, no protein; lane 2, ad libitum (30 μg); lane 3, ad libitum (60 μg); lane 4, fasting (60 μg); lane 5, refeeding (60 μg); lane 6, no protein. Nucleotide numbers refer to the positions with respect to the previously reported ATG codon as position +1. The protected regions are indicated by boxes with their names and positions on the promoter sequence (Box I to III), and putative DR+1 sequence was underlined.

4. PPARγ binds to the mouse GLUT2-PPRE

In the protected region shown in DNase I footprinting assay, it was found that this sequence includes the distinct DNA sequence elements called DR+1 sites, which is known to bound PPARγ and RXRα heterodimer (Fig. 4). To determine whether PPARγ and RXRα heterodimer activate mouse GLUT2 promoter through direct DNA binding, we performed EMSA using GLUT2-PPRE (-197/-184 fragment) as a probe which contains DR+1 sequence. As shown in Fig. 5, the addition of nuclear extract resulted in two shifted DNA-protein complex clearly. The fast migrating band was displaced by the addition of unlabeled CYP4A6 oligonucleotides containing known PPARγ consensus sequence in dose dependent manner (Fig. 5, lane 3, 4, and 5). Also, binding of this DNA-protein complex was attenuated when anti-PPARγ antibody was added to the reactions in the concentration from 0.1 to 1 ㎕ (Fig. 5, lane 6, 7, 8, 9, and 10), suggesting that the fast migrating bands contain the heterodimer of endogenous PPARγ and RXRα. In order to investigate the characteristics of protein binding further, EMSA using mutated probe was carried out. Since GLUT2-PPRE (-197/-184 fragment) has two hexanucleotide repeats (DR+1) in its sequence, we first tested mutated competitors (M1 and M2) for DR+1 sequence (Fig. 6A). This arrangement of mutations has been proven to be effective in abolishing PPARγ binding to the

PPRE. As compared to wild type competitors (Fig. 6B, lane 3), the addition of M1 or M2 resulted in the reappearance of slow migrating band, whereas fast migrating band was still competed out. Because M1 did not show significant difference from wild type competitor (Fig. 6B, lane 5), it is suggested that PPARγ binds to the downstream region of M2. When another mutation (M3) was introduced along with M2 mutation, the DNA-protein binding complex was not interfered by the addition of competitor, suggesting that PPARγ bind to M2 and M3 region. Same results were obtained when mouse liver nuclear extracts were used (Fig. 6B, lane 9 to 15). Using MatInspector V2.2 based on TRANSFAC 4.0 software (German Research Centre For Biotechnology, German), it was suggested the presence of C/EBP binding site in the GLUT2-PPRE, so we tested the binding of C/EBPα and C/EBPβ. However, competition and supershift assay for C/EBP did not suggest any C/EBP binding (Fig. 7).

Because the EMSA experiments indicated that individual mutations in the M1, M2, or M3 region on GLUT2-PPRE regions failed to result in the responsiveness of PPARγ, we introduced these mutations to the promoter-luciferase construct, pMGT2m-203E1 (M1) and pMGT2m-196EI (M2), and examined the promoter activity. In Alexander cells, M1 mutation did not affect the promoter activity by PPARγ overexpression. However, in case of

pMGT2m-196, the promoter activity by PPARγ was dramatically decreased (Fig. 8). Taken together, it is suggested that both M2 and M3 are important for binding of PPARγ even though there was little sequence similarity with conventional DR+1.

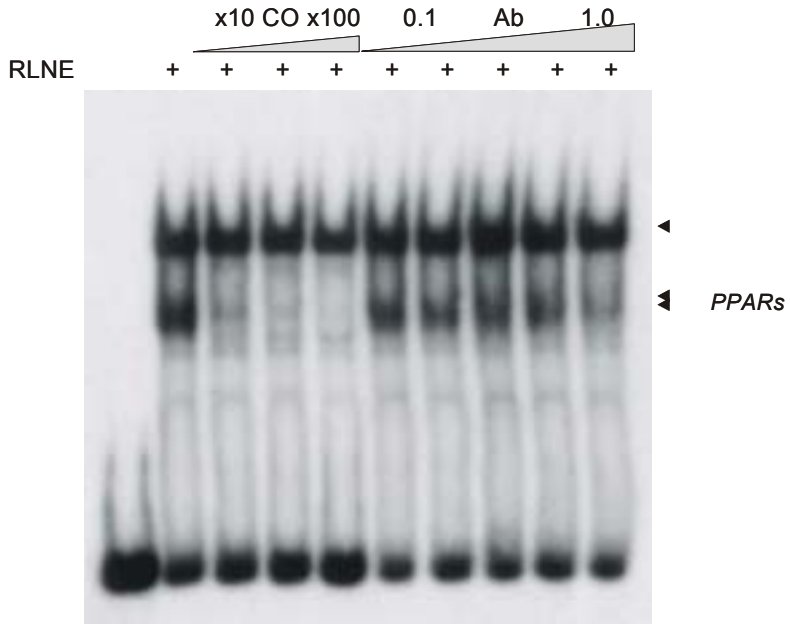


Fig 5. EMSA with competitor and anti-PPARγ antibody in mouse GLUT2 PPRE. EMSA was performed with 5 μg of rat liver nuclear protein (RLNE) and indicated amount of unlabeled CYP4A6 consensus (CO) and anti-PPARγ (Ab). The probes was used to 50,000 cpm of [^{32}P]-labeled MGT2-PPRE. The upper arrow indicates the unknown binding complexes, whereas the lower arrow indicates the position of the PPARγ complex.

A.

DR1 AGGTCANAGGTCA

DR1' AGGTCANAGGTCA

CTGTGCTCAAGCCACAAGTCATTGGGGTAAAGGGT

M1 CTGTGCTC**GAATTC**CAAGTCATTGGGGTAAAGGGT

M2 CTGTGCTCAAGCCAC**GAATTC**TTGGGGTAAAGGGT

M3 CTGTGCTCAAGCCACAAGTCAT**GAATTC**AAAGGGT

M2+3 CTGTGCTCAAGCCAC**GAATTC**T**GAATTC**AAAGGGT

B.

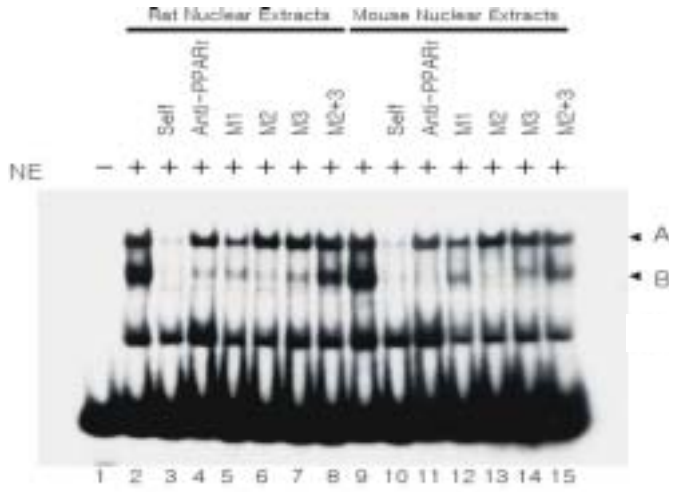


Fig. 6. Effect of mutated competitors on the binding of PPARγ/RXRα to GLUT2-PPRE. A. Site-directed mutations were introduced into GLUT2-PPRE oligonucleotides. This arrangement of mutations has been proven to be effective on abolishing PPARγ binding to the PPRE. M1, M2, M3, and M2+3 are mutations designed to disrupt the consensus binding site derived from the known PPREs. The position of potent mouse GLUT2 PPRE sequence were compared with DR1, as indicated by DR1 and DR1'. B. EMSA was performed with GLUT2-PPRE as a probe on 4% (w/v) non-denaturing polyacrylamide gel. Thirty pmole of each double-stranded oligonucleotide with 5 μg of rat or mouse liver nuclear extracts were used in these experiment. NE: Liver Nuclear Extract; Competitor: self, M1, M2, M3, and M2+3; Antibody: anti-PPARγ. A and B arrows indicate the protein-DNA complexes.

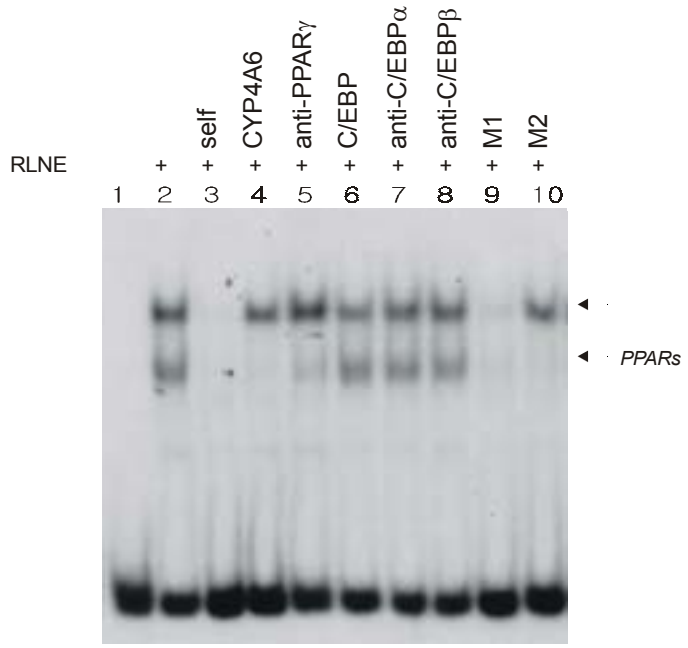


Fig. 7. Analysis of PPARs and C/EBPs binding to mouse GLUT2-PPRE. EMSA was performed with GLUT2-PPRE containing putative binding site of C/EBP as a probe on 4% (w/v) non-denaturing polyacrylamide gel. Thirty pmole of each double-stranded oligonucleotide with 5 ㎍ of rat or mouse liver nuclear extracts were used in these experiment. RLNE: Rat Liver Nuclear Extract, ad libitum, 5 ㎍; Competitors: self, CYP4A6, M1, M2; Antibody: anti-PPARɣ, anti-C/EBPα and β. The DNA-protein complexes are indicated by arrows.

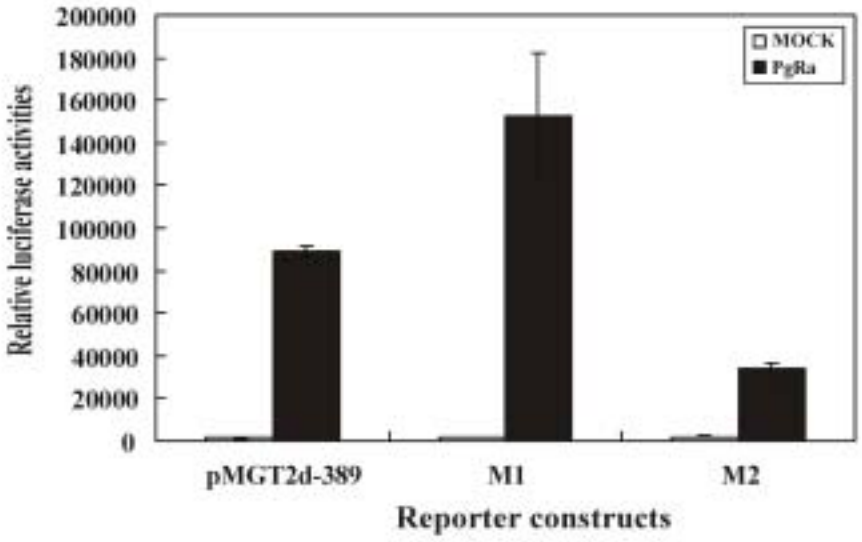
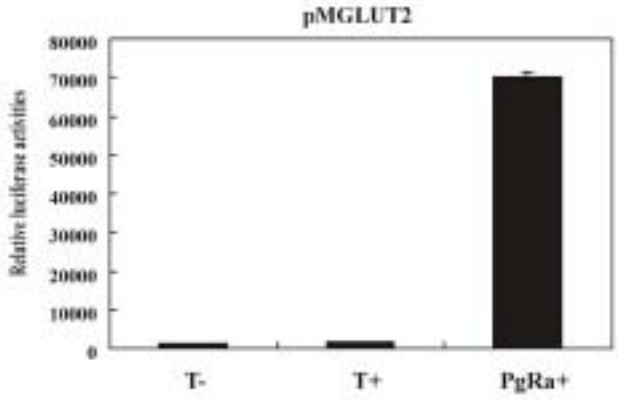


Fig. 8. Effect of mutations in the putative PPRE region of the mouse GLUT2 promoter in Alexander cell. M1 and M2 mutations were introduced to luciferase constructs and designated as M1 and M2. The reporter constructs containing a wild and mutant sequence of mouse liver GLUT2 promoter were transfected into Alexander cells. Luciferase activities were normalized on the basis of β-galactosidase activity. Results are the means ± S.D. of three independent experiments in a triplicate.

5. GLUT2 mRNA level was increased in mouse primary hepatocytes by PPARγ

From the experiments described above, it is thought that PPRE on mouse GLUT2 promoter could be important in regulating the gene expression in vivo. If PPARγ and troglitazone can activate the mouse GLUT2 promoter through GLUT2-PPRE, then GLUT2 expression should be increased by PPARγ and troglitazone in primary hepatocytes. To examine this possibility, we performed RT-PCR as well as transient transfection study using mouse primary hepatocytes, with or without PPARγ overexpression. In the condition which PPARγ transactivates GLUT2 promoter (Fig. 9A), the endogenous GLUT2 mRNA amount was increased following the induction of PPARγ and treatment of troglitazone in primary hepatocytes (Fig. 9B). Interestingly, treatment of troglitazone alone failed to increase GLUT2 mRNA expression. This result strongly support the idea that PPARγ is the transcriptional regulator of GLUT2 expression in vivo.

A.



B.

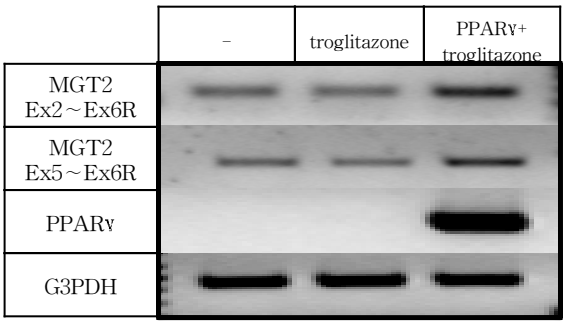


Fig 9. The effect of troglitazone and PPARγ in endogenous GLUT2 expression in mouse primary hepatocytes. A. Activation of luciferase in the primary hepatocytes by receptor ligand complex. Ligands; 20 μM troglitazone, 1 μM 9-*cis* retinoic acid, Receptor; PPARγ/RXRα (1 μg each). B. RT-PCR of GLUT2. Total RNAs were isolated from same groups of mouse primary hepatocytes used in the experiment shown in A. G3PDH was used as an internal control.

Ⅳ. Discussion

The PPARγ/RXRα heterodimer is known to bind to DR+1 sequence. The binding of ligands to nuclear receptors is known to stimulate release of negative factors (corepressors) and trigger binding of positive cofactors (coactivators), so as to regulate the expression of many genes involved in metabolism.[31,32] For example, in adipose tissue of type 2 diabetes, regulation of glucose transporter type 4 and insulin signaling were regulated by troglitazone.[33]

The expression of GLUT2 in mouse liver could be regulated by PPARγ. It was reported that mouse GLUT2 was overexpressed in the liver of ob/ob mouse by troglitazone and cause of a decrease in blood glucose level.[34] From these findings, the mouse GLUT2 promoter have been thought to be a target gene of PPARγ, which may play an important role in the regulation of blood glucose level. Moreover, it was reported that the mRNA for the glucose transporter GLUT2 was decreased by 64% in the fasted and increased by 93% in the fed state hepatocytes.[35] This provides important features of glucose homeostasis because it was also reported that PPARγ mRNA levels are decreased by 50% during fasting in both white and brown adipose tissue. Therefore, it is possible that fasting may strongly influence PPARγ expression

not only in adipose tissues but also in liver.[36] These results suggest that hepatic PPARγ is involved in regulation of blood glucose levels.

We showed that PPARγ directly bound to PPRE on GLUT2 promoter by EMSA. Although this site contains putative PPARγ/RXRα binding sequences, there was little sequence similarity with conventional DR+1. PPARs bind to cognate DNA elements called PPREs in the 5' flanking region of target genes. Known PPREs are direct repeats of an AGGNCA half-site separated by a one-base pair spacer. A short sequence located immediately upstream of the first half-site confers polarity on the PPRE, with the PPAR moiety binding 5' to the RXR half of the heterodimer.[37,38] The gel mobility shift assay showed the direct binding of PPARγ-retinoid X receptor complex to the PPRE. There are reports of atypical PPREs found on many PPAR regulating genes[37], hence some investigators have examined the binding property of previous known PPREs, reporting that PPAR can bind to and activate variety of atypical PPREs with variable affinity. In this regard, it is noteworthy that the PPRE found in the GLUT2 promoter showed two shifted DNA-protein complex in EMSA experiment. The addition of M2 or M3 mutant competitor resulted in reappearance of slow migrating complex, suggesting that this PPRE may bound another transcription factor other than PPARγ/RXRα complex. Moreover, M2 mutation alone in GLUT2 promoter-luciferase construct showed dramatic

decrease in response to PPARγ overexpression, suggesting that unknown transcription factor or coactivator could be interacting with PPARγ/RXRα complex.

GLUT2 is main glucose transporter of liver, and has been emphasized to be important in fasting/feeding regulation of glucose metabolism as well as in diabetes.[35] There are many unknown mechanisms by which transcriptional regulation could be achieved in tightly controlled manner in physiological and disease status. The role of PPARγ in liver, together with its effect on GLUT2 expression, could be a target for understanding glucose metabolism of living organisms.

Ⅴ. Conclusion

We have localized the PPRE (-197/-184) in the mouse GLUT2 promoter. The region was regulated by PPARγ, which may play an important role in the regulation of blood glucose level. Troglitazone treatment resulted in the increase in GLUT2 mRNA in the isolated hepatocyte system. From these results, troglitazone may play an important role in regulating blood glucose level by activating hepatic GLUT2 level.

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Abstract (In Korea)

마우스 간장에서 PPARγ에 의한 간장형 포도당운반체 promoter의 조절

간장형 포도당운반체는 간장과 췌장 베타세포에서 주로 발현되며 생체 내에서 당질의 항상성을 유지하는데 중요한 역할을 한다. 간장형 포도당운반체의 유전자 발현이 여러 간장 특이 전사인자에 의해 조절된다는 보고가 있으며, 또한 PPARγ가 간장에서 당질대사에 관여하는 유전자들의 발현을 조절한다고 알려져 있다. 따라서 본 실험에서 PPARγ가 포도당운반체 유전자 조절에 어떤 영향을 미치는지 조사하였다.

PPARγ을 과발현시킨 경우에 사람, 백서, 마우스의 포도당운반체 유전자 promoter 활성을 조사한 결과 마우스에서 troglitazone에 의한 가장 높은 활성을 보였다. 이런 활성의 증가는 마우스의 일차배양 간장세포 뿐만 아니라 여러 가지 다른 세포주에서도 같은 경향을 나타내었다. Promoter의 염기서열을 순차적으로 제거한 후 활성을 측정한 결과 -166까지 제거한 경우 활성이 크게 낮아지는 것을 볼 수 있었다. 또한 백서의 간장 핵단백질을 이용한 DNase I footprinting 실험에서 -206에서 -147 부위에 단백질이 결합하는 것을 확인하였다. 이 부위에 PPARγ가 결합하는지를 확인하기 위하여 EMSA를 수행한 결과 -197/-184 부위에 PPRE가 존재한다는 것을 밝힐 수 있었다. 또한 이 부위를 돌연변이 시켰을 때 promoter의 활성이 급격하게 떨어지는 것을 관찰하였다. 이상의 결과는 PPARγ가 마우스 간장세포에서 간장형 포도당운반체 mRNA의 발현을 증가시킬 수 있다는 것을 보여주며, 마우스 간장형 포도당운반체 promoter가 PPARγ에 의해 조절되는 기전과 troglitazone을 제 2형 당뇨병 환자에게 투여하였을 때 어떻게 혈당이 조절되는지를 설명하는 것이라 할 수 있다.

핵심되는 말: 간장형 포도당운반체, PPARγ, troglitazone, 간장, 간장세포