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Transcriptional Regulation of Aquaporin 3 by Insulin

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Abstract In the current study, we identified a regulatory factor for the transcription of aquaporin 3 (AQP3) whose expression is repressed by insulin. We constructed a luciferase reporter vector containing bp −1382 to −12 of the 5′-flanking region of the AQP3 gene for a reporter gene assay and observed that luciferase activity in transfectants with the plasmid decreased on treatment with insulin. Serial deletion constructs revealed two regions responsible for the insulin-mediated repression, one between bps −1382 and −780, and the other between bps −404 and −82. mRNA expression of forkhead box a2 (Foxa2), the binding site of which was located between bps −1382 and −780, was found to decrease on treatment with insulin. A mutant reporter plasmid with an altered Foxa2-binding site and siRNA for the Foxa2 sequence counteracted the insulin-mediated repression of AQP3 transcription. These results suggest that Foxa2 is one of the transcriptional regulators for AQP3 gene expression regulated by insulin. J. Cell. Biochem. 102: 1051–1058, 2007. © 2007 Wiley-Liss, Inc.

Key words: aquaporin 3; insulin; forkhead box a2; gene expression; transcriptional regulator

Thirteen molecular species of aquaporin (AQP) have been found in human tissues. They have been numbered AQP0 to AQP12 in order of discovery. AQPs are broadly classified into two groups: AQP0, 1, 2, 4, 5, 6, and 8 and AQP3, 7, 9, 10, 11, and 12, by their physiological functions [Ishibashi, 2003]. The former group is permeable by water only while the latter, known as aquaglyceroporins, is permeable not only by water, but also by water-soluble low-molecular weight molecules such as glycerol and urea. Although AQPs are widely distributed in almost all human tissues, they show a specific localization [Ishibashi, 2003]. AQP3 is expressed in the human gastrointestinal tract, red blood cells, the kidney, skin, and the brain and extensively on the basal membrane of epithelial cells in the ileum and colon [Silberstein et al., 1999]. The physiological functions of AQP3 in the kidney, gastrointestinal tract, and skin have been discussed based on observations in AQP3-deficient mice. AQP3 may be involved in renal failure caused by nephrogenic diabetes insipidus and hydronephrosis, the absorption of glycerol resulting from a decrease in the level of glycerol in serum, skin hydration from the promotion of dry skin, and the occurrence of diarrhea and constipation [Hara et al., 2002; Ishibashi, 2003].

AQP7 and 9, mainly expressed in adipocytes and the liver, respectively, are classified as aquaglyceroporins similar to AQP3. One physiological function of these AQPs may be to take glycerol in and out of tissues: glycerol is released by AQP7 from adipocytes and taken up into the liver by AQP9. Recently, insulin was found to regulate the expression of AQP7 and 9 [Kishida et al., 2000, 2002; Kuriyama et al., 2002; Carbrey et al., 2003]. It was suggested that these AQPs took part in the control of blood glucose level through the regulation of glycerol release from adipocytes and glycerol intake to liver. The level of gluconeogenesis utilizing glycerol was also thought to increase in insulin registrants due to a failure to control the transport of glycerol through AQP7 and 9 down-regulated by insulin [Kuriyama et al., 2002].

It seems appropriate to consider AQP3 responsible for the absorption of glycerol in the organs of the digestive system because glycerol

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TABLE I. Sequences of Oligonucleotide Primers and Assay Conditions for PCR

Gene name	Sequence	Product size (bp)	PCR condition	Cycle number
AQP3	Forward: 5′-GGAATAGTTTTTGGGCTGTA-3′; reverse: 5′-GGCTGTGCCTATGAACTGGT-3′	159	94°C, 30 s; 57°C, 30 s; 72°C, 1 min	27
Foxa2	Forward: 5′-AGGAGGAAAACGGGAAAGAA-3′; reverse: 5′-CTGCAACAACAGCAATGGAG-3′	137	94°C, 30 s; 55°C, 30 s; 72°C, 30 s	25
C/EBPα	Forward: 5′-TGTATGCCCCTGGTGGGAGA-3′; reverse: 5′-TCATAACTCCGGTCCCTCTG-3′	164	94°C, 30 s; 52°C, 30 s; 72°C, 1 min	29
SP1	Forward: 5′-GGCTACCCCTACCTCAAAGG-3′; reverse: 5′-CACAACATACTGCCCACCAG-3′	103	94°C, 30 s; 52°C, 30 s; 72°C, 1 min	29
CREB	Forward: 5′-GGAGCTTGTACCACCGGTAA-3′; reverse: 5′-GGGCTAATGTGGCAATCTGT-3′	136	94°C, 30 s; 58°C, 30 s; 72°C, 1 min	29
β-Actin	Forward: 5′-GAGGTACTCCGTGTGGATCG-3′; reverse: 5′-AGTCCGCCTAGAAGCATTTG-3′	131	94°C, 30 s; 60°C, 30 s; 72°C, 1 min	20

is involved in the digestion of nutrients and AQP3 is permeable by glycerol. We speculate that the expression of AQP3 is regulated by insulin like that of AQP7 and 9. Then, we previously reported that insulin repressed transcription of the AQP3 gene in Caco-2 cells and that the signal transducers phosphoinositide 3 kinase (PI3K) and the mammalian target of rapamycin (mTOR) were involved in the signal pathway regulating the transcription [Asai et al., 2006]. In the present study, we demonstrate that a transcriptional regulator, forkhead box a2 (Foxa2), is responsible for AQP3 gene expression repressed by insulin.

MATERIALS AND METHODS

Materials

Recombinant human insulin was purchased from Wako Pure Chemicals, Ltd. (Osaka, Japan). TRIzol, the Electrophoretic Mobility Shift Assay (EMSA) kit, the Oligo $(dT)_{12-18}$ Primer, and SuperScript III reverse transcriptase were obtained from Invitrogen Corp. (Carlsbad, CA). TaKaRa EX Taq and TaKaRa Taq, and FuGene6 were purchased from Takara Bio Inc. (Osaka, Japan) and Rosch Diagnostics K. K. (Tokyo, Japan), respectively. The Dual-Luciferase Reporter Assay System, the GeneEditor in vitro Site-Directed Mutagenesis System, and other DNA-manipulating enzymes were acquired from Promega Corp. (Madison, WI). siRNAs for the Foxa2 sequence and a negative control were purchased from Ambion Inc. (Austin, TX). All other chemicals and reagents were of analytical grade.

Cell Culture and Treatment With Insulin

Caco-2 cells were maintained in Dulbeccos Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells were plated on a 35-mm dish at a density of 3×10^6 cells/ml and incubated in a CO_2 incubator for 36 h. Cells were washed once with phosphate buffered saline (PBS) then treated with insulin dissolved in serum-free DMEM containing 0.5% bovine serum albumin (BSA) in a CO_2 incubator.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for AQP3 and β-Actin

Total RNA was extracted from Caco-2 cells with TRIzol reagent. First-strand cDNAs were prepared from 5 μg of total RNA by SuperScript III reverse transcriptase and used as a template. RT-PCR was performed with the primers listed in Table I under optimal conditions. PCR products were resolved on a 1.5% agarose gel and visualized with ethidium bromide under UV light. Band intensity was quantified using the Scion Image program. Real-time RT-PCR was performed with the specific primers for AQP3 (Table I) and SYBR Premix Ex *Taq* (Takara) using a iCycler-iQ real-time PCR equipment (Bio-Rad).

Western Blotting

Caco-2 cells treated with or without insulin were lysed with 1% sodium dodecyl sulfate (SDS) and 4 mM 2-mercaptoethanol.

SDS-polyacrylamide gel electrophoresis was carried out with a 12% polyacrylamide gel according to the method of Laemmli [1970]. Western blotting was performed with goat anti-AQP3 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and anti-goat IgG peroxidase conjugate (Sigma–Aldrich, Saint Louis, MO) using the method of Towbin et al. [1979]. Peroxidase activity was detected using an ECL Western Blotting Detection System according to the manufacturer's instructions (Amersham Biosciences).

Construction of Reporter Vector

The genomic DNA in Caco-2 cells was extracted with TRIzol reagent according to the manufacturer's instructions and used as a template for PCR amplification. A DNA fragment containing the regulatory region −1382 to −12 5′-upstream of AQP3's transcription initiation site was prepared by PCR with a forward primer containing a *Kpn*I recognition site, 5′-CAGGTACCCGAGGCTACAGTGAGCTGTG-3′, and a reverse primer containing a *Xho*I recognition site, 5′-CGCTCGAGAGCGCTGGTGGCTCCCTTTA-3′. After the PCR product was digested with both restriction enzymes, the fragment was ligated into a multiple cloning site of pGL3 basic vector and the plasmid obtained was named 1382pGL3. A serial deletion of the fragment ligated into the vector was performed with *BAL*31 nuclease, after 1382pGL3 was digested with *Kpn*I. The deletion with the nuclease was terminated by extracting the deleted fragments with phenol–chloroform–isoamyl alcohol and the fragments were ligated into pGL3 basic vector after being treated with T4 DNA polymerase and subsequently with *Hin*dIII. The constructed plasmids which contained the fragments spanning bp −780 to −12 and −404 to −12 were named 780pGL3 and 404pGL3, respectively. The PCR product was treated with *Pst*I and *Hin*dIII and the digested fragment bp −82 to −12 was ligated into pGL3 basic vector, yielding 82pGL3.

Site-directed Mutagenesis

Site-directed mutagenesis in the Foxa2-binding element was performed with the GeneEditor in vitro Site-Directed Mutagenesis System according to the manufacturer's instructions. Five micrograms of 1382pGL3 as a template DNA and an oligonucleotide for the mutagenesis, 5′-AATCCCCATCTCCACTAAAAGCGTCAAAATTAGCGTCAAAATTAGCTGGGCATGGTG-3′, were used. The mutated plasmid obtained was named $Foxa2_{mut}$pGL3.

Transcriptional Analysis

Caco-2 cells were plated onto a 24-well culture plate at a density of 5×10^4 cells/well and cultured for 4 h in a CO_2 incubator. After attaching to the plate, the cells were transfected with 0.4 μg of the reporter vectors prepared above and 5 ng of phRL-TK as a reference plasmid with 1.2 μl of FuGene6 according to the manufacturer's instructions. The activities of luciferases from the firefly and *Renilla* were detected with the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions.

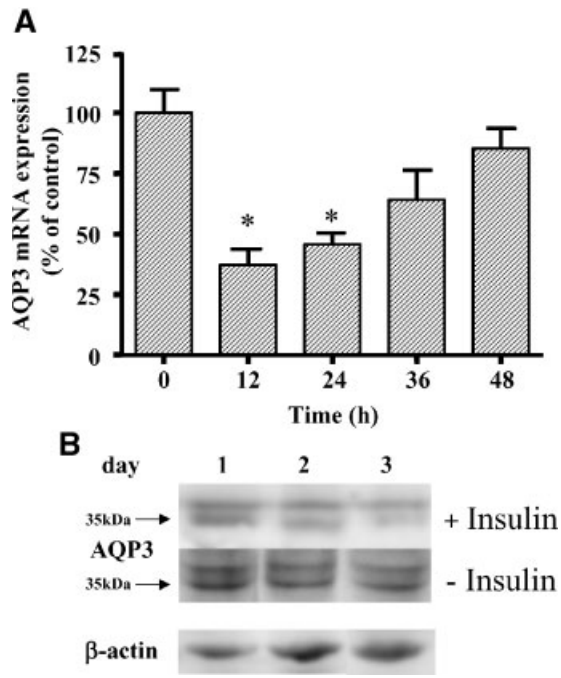

Fig. 1. Effect of insulin on mRNA and protein expression of AQP3 in Caco-2 cells. **A**: Caco-2 cells were incubated with 100 nM insulin for the period indicated. After total RNA was extracted, the expression of mRNA was quantified by the real-time RT-PCR method. The results were normalized with the β-actin mRNA levels and the mRNA level of the control was taken as 100%. Data show the mean ± S.D. of five experiments. **B**: Caco-2 cells were cultivated with or without 1 μM insulin for the period indicated. Cell lysates were prepared with lysis buffer containing SDS and 2-mercaptoethanol and separated by electrophoresis with a 12% SDS-polyacrylamide gel. After proteins in the gel were electroblotted on a PVDF membrane, AQP3 and β-actin were probed with anti-AQP3 and anti-β-actin antibodies, respectively, and visualized using a second antibody-peroxidase conjugate and the ECL system. $^*P < 0.05$, versus control.

Caco-2 cells (3.5×10^5) were transfected with 0.09 nmole of small interfering RNA (siRNA) for Foxa2 and a negative control sequence (Ambion, Inc., Foster, CA, Catalog #116312 and #4611, respectively) by using siPORT NeoFX (Ambion) according to the manufacturer's instructions after annealing in the supplied buffer Opti-MEM I medium. Transfected cells were maintained in regular culture medium for a day and treated with insulin in serum-free DMEM containing 0.5% BSA.

Electrophoretic-mobility Shift Assay (EMSA)

EMSA was performed with the EMSA kit according to the manufacturer's instructions. Two sets of oligonucleotide probes, Foxa2(+), 5′-AAAAAAACAAAAATTAGCTG-3′, and Foxa2(−), 5′-TTTTTTTGTTTTTACGAC-3′, and mFoxa2(+), 5′-AAAAGCGTCAAAATTAGCTG-3′, and mFoxa2(−), 5′-TTTTCGCAGTTTTAA-TCGAC-3′, were used. The complex of oligonucleotide and protein was resolved by electrophoresis with a 6% non-denaturing polyacrylamide gel. Nucleotides in the gel were visualized with SYBER Green EMSA supplied with the kit. Protein was assayed by the method of Bradford [1976].

Statistical Analysis

The significance of differences between two groups was calculated with Student's *t*-test, and the significance of differences between multiple groups was assessed by an one-way analysis of variance followed by Dunnet's test.

RESULTS

Effect of Insulin on mRNA and Protein Expression of AQP3

The mRNA and protein expression of AQP3 in Caco-2 cells treated with an approximately physiological concentration of insulin was investigated by RT-PCR. The mRNA expression was observed to decrease significantly after treatment of the cells with 100 nM of insulin for 12 and 24 h (Fig. 1A) and the protein expression

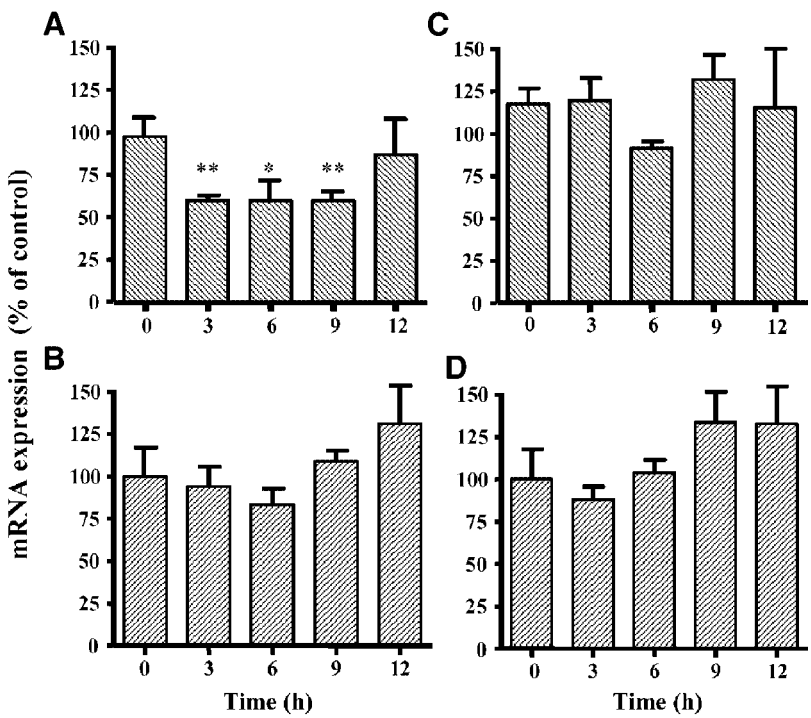

Fig. 2. Effect of insulin on mRNA expression of Foxa-2, C/EBPα, CREB, and Sp1 in Caco-2 cells. Caco-2 cells were incubated with 0.1 μM insulin for the period indicated. Total RNA was isolated and subjected to RT-PCR analysis using specific primers for **A**: Foxa2, **B**: C/EBPα, **C**: CREB, and **D**: Sp1. The products were resolved on 1.5% agarose gels and visualized with ethidium bromide. mRNA expression was quantified to measure the band intensity using the Scion Image program. The intensities of Foxa2, C/EBPα, CREB, and (D) Sp1 were normalized with that of β-actin mRNA and the mRNA level of the control was taken as 100%. Data represent the average ± S.D. from three experiments. $^*P < 0.05$, $^{**}P < 0.01$ versus 0 h.

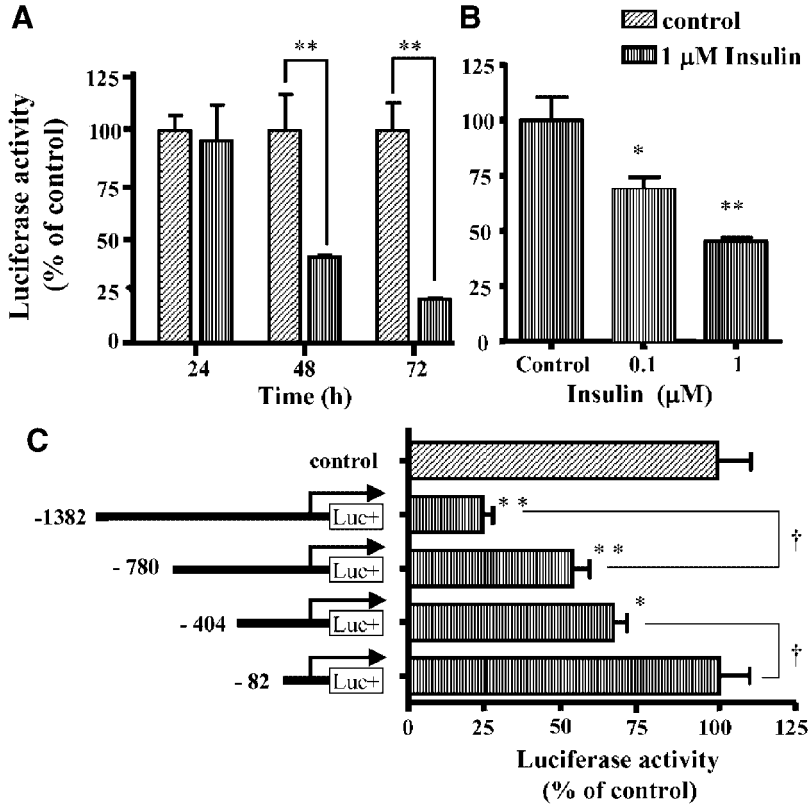

Fig. 3. Effect of insulin on the promoter activity of AQP3 in Caco-2 cells and promoter activity of a series of constructs with deletions of the 5′-flanking region of the AQP3 gene. Caco-2 cells were simultaneously transfected with a plasmid vector containing the promoter region (bp −1382 to −12) of AQP3 and a firefly luciferase reporter gene and the vector phRL-TK vector containing a *Renilla* luciferase gene. **A**: Caco-2 cells were incubated with 100 nM insulin for the period indicated. **B**: Caco-2 cells were incubated with or without various concentrations of insulin for 48 h. **C** Serial deletion constructs were prepared to ligate various sized regions of the AQP3 promoter to the reporter gene vector containing a firefly luciferase reporter gene. Caco-2 cells were simultaneously transfected with these plasmid vectors and phRL-TK containing a *Renilla* luciferase gene and incubated with 1 μM insulin for 48 h. Cell lysates were prepared after incubation and luciferase activities were measured with a Dual-Luciferase Reporter Assay System. Firefly luciferase activity was normalized with *Renilla* luciferase activity and is expressed relative to the control. Data represent the average ± S.D. from six experiments. $*P < 0.05$ versus control. $**P < 0.01$ versus control. $\dagger P < 0.05$ 1382pGL3 versus 780pGL3 and 404pGL3 versus 82pGL3.

also decrease 48 and 72 h after treatment with 1 μM of insulin (Fig. 1B).

Foxa2 mRNA Expression Suppressed by Insulin

The binding sites of four transcriptional regulatory factors, C/EBPα, Foxa2, CREB, and Sp1, which were related to the signal transduction pathway of insulin, were found in the promoter region of AQP3 from bp −1382 to −12 5′-upstream of the transcription initiation site as a result of searching the TSFSEARCH database. Whether insulin affected mRNA levels of these factors was examined by RT-PCR with specific primers (Fig. 2). The expression of Foxa2 was found to decrease significantly with time when the Caco-2 cells were treated with 0.1 μM insulin. Other factors were not responsive to the insulin treatment.

Promoter Activity of the AQP3 Gene

AQP3's promoter region from bp −1382 to −12 5′-upstream of the transcription initiation site was subcloned into the luciferase reporter vector pGL3, and this construct, 1382pGL3, was introduced into Caco-2 cells to determine whether the promoter activity of the AQP3 gene was affected by insulin (Fig. 3A,B). Luciferase activity significantly decreased to less than 50% of the control level on treatment with 1 μM insulin for 48 and 72 h, and 0.1 and 1 μM insulin for 48 h. This observation of reduced AQP3 promotor activity after 48 h treatment with

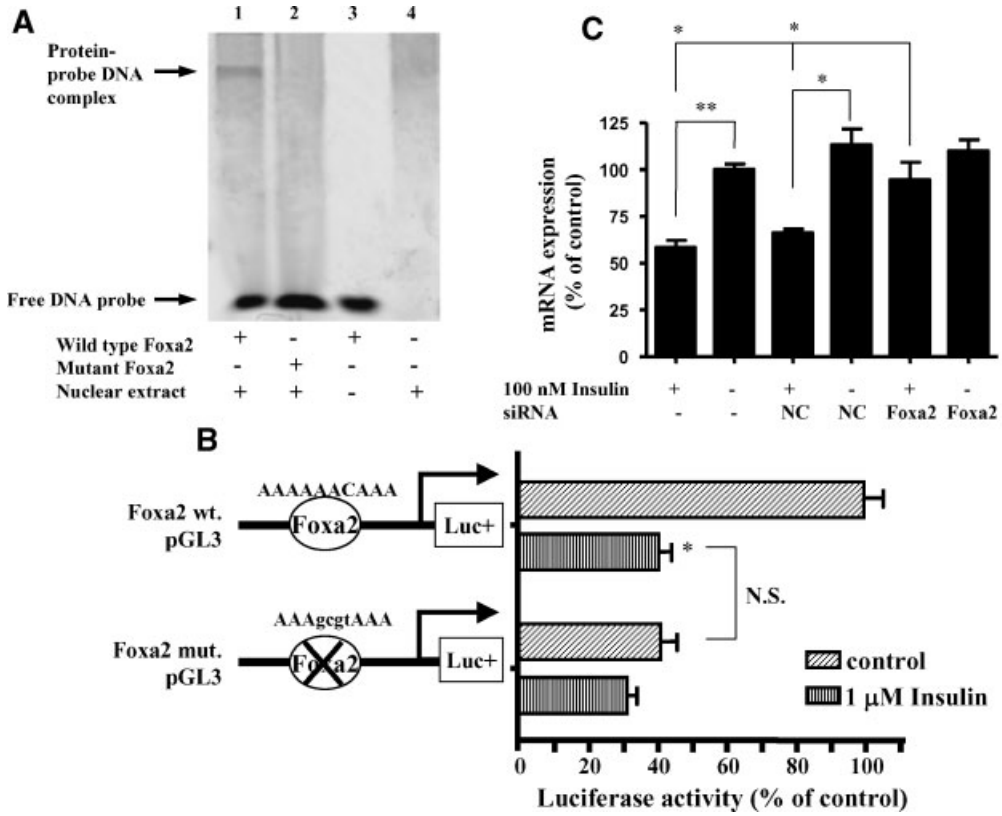

Fig. 4. Transcriptional regulation of AQP3 gene by Foxa2. **A**: EMSA for consensus and mutated Foxa2 sequences in the AQP3 promotor region with Caco-2 nuclear extract. Caco-2 nuclear extracts (6 μg) were incubated with consensus (lane 1) and mutated (lane 2) sequences of Foxa2 as probes or without a probe (lane 4). A consensus Foxa2 sequence alone (lane 3) was also prepared. The reaction products were resolved in a 6% nondenatured polyacrylamide gel and visualized with a FLA-5100 digital photograph system. **B**: Effect of mutation of the Foxa2-binding sequence in the AQP3 gene promoter on insulin-mediated repression. Wild-type and mutant constructs of the AQP3 gene promoter were ligated to a luciferase vector. Four base pairs were mutated in the Foxa2-binding sites. Caco-2 cells were simultaneously transfected with a plasmid containing the consensus sequence of Foxa2 or a mutated sequence and phRL-TK containing a *Renilla* luciferase gene and incubated with 1 μM insulin for 48 h. Cell lysates were prepared after incubation and luciferase activities were measured with a Dual-Luciferase Reporter Assay System. Firefly luciferase activity was normalized with *Renilla* luciferase activity and is expressed relative to the control. Data represent the average ± S.D. from six experiments. **C**: Effect of siRNA for the Foxa2 sequence on insulin-mediated repression of the AQP3 gene. Caco-2 cells were incubated with 100 nM insulin for 12 h in a serum-free medium containing 0.5% BSA after being transfected with siRNAs for Foxa2 and a negative control (NC) sequence. The mRNA expression was quantified by the real-time RT-PCR method. The results were normalized with the β-actin mRNA levels and the mRNA level of the control was taken as 100%. Data show the mean ± S.D. of five experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

insulin seems to be inconsistent with the result shown in Figure 1 that less than 50% reduction of AQP3 mRNA abundance was observed already 12 h after addition of insulin. However, this discrepancy may be solved by the inference that enough amount of luciferase protein for measuring the activity is accumulated for 48 h in transfected cells.

Promoter activities of a series of constructs with deletions in the AQP3 promoter region were assayed to define the specific region required for the repression of AQP3's transcription by insulin. As shown in Figure 3C, the transcription was significantly restored when the regions between bps −1382 and −780 and between bps −404 and −82 were removed although the deletion of bp −780 to −404 did not affect the promoter activity of the AQP3 gene.

AQP3's Transcription Regulated by Foxa2

An electrophoretic-mobility shift assay was performed to confirm the specific binding of the protein to the Foxa2-binding site in the promoter region of AQP3 (Fig. 4A). Although the specific complex binding with the consensus probe containing the mature Foxa2-binding sequence was detected, no specific binding with the mutant probe was observed.

Since one of the transcriptional regulatory factors for the AQP3 gene was predicted to be Foxa2 from the results shown in Figures 2 and 4, it was examined whether the Foxa2 mutant constructed by site-directed mutagenesis is responsible for the repression of AQP3's transcription by insulin (Fig. 4B). The promoter activity of the mutant was observed to decrease significantly by about 40% and the repression by insulin disappeared.

To further confirm that Foxa2 participates in the transcriptional control of the AQP3 gene, Caco-2 cells were transfected with a siRNA targeting the Foxa2 sequence and it was determined whether the expression of AQP3 mRNA was suppressed by insulin. As shown in Figure 4C, the down-regulation of AQP3 gene expression by insulin was observed to disappear on transfection of the siRNA into Caco-2 cells.

DISCUSSION

We demonstrated that a transcription factor, Foxa2, was one of the regulators responsible for transcription of the AQP3 gene in Caco-2 cells. Insulin showed to repress transiently the mRNA expression of Foxa2 in Caco-2 cells after 3–9 h. The introduction of a mutation of the Foxa2-binding sequence in the promoter region of AQP3 and siRNA for the Foxa2 sequence resulted in a loss of insulin-mediated repression for transcription of the AQP3 gene. These results suggest that AQP3 contributes to the hypoglycemic effect of insulin since AQP3 is permeable by glycerol, a resource for gluconeogenesis and triglyceride synthesis.

Foxa2, previously known as hepatocyte nuclear factor 3-β (HNF3β), was reported to regulate the transcription of the genes for lipase, calnitine palmitoyltransferase 1, acyl CoA dehydrogenase, phosphoenolpyruvate carboxylase, and glucose 6 phosphatase related to glycometabolism and lipid metabolism in the liver [O'Brien et al., 1995; Shih et al., 1999; Kaestner, 2000; Wolfrum et al., 2004]. Recent studies revealed that Foxa2 was not only involved in the transcriptional regulation of enzymes participating in fatty acid oxidation in the liver but also a potassium channel triggering secretion of insulin in the pancreas [Wang et al., 2002] and essential for the development of both the liver and pancreas [Duncan et al., 1998; Lee et al., 2005]. Insulin-receptor signaling was reported to activate signal transducers in the pathway of PI3K-Akt, trigger phosphorylation of ^{156}Thr in Foxa2, and result in the nuclear export of Foxa2 [Wolfrum et al., 2003]. Therefore, Foxa2 is thought to lose its transcriptional activity because of inaccessibility to the binding site of the target DNA. We found a significant decrease in the level of Foxa2 mRNA on exposure to insulin a few hours before the repression of AQP3 expression mediated by insulin, suggesting that Foxa2 takes part in the transcription of the AQP3 gene, although the intracellular localization of Foxa2 was unclear.

We demonstrated that Foxa2 participated in the transcriptional regulation of the AQP3 gene in the insulin-mediated suppression of AQP3 expression. Constructs lacking the Foxa2-binding sequence were still responsive to the insulin-mediated repression of the AQP3 gene and, as shown in Figure 3, the magnitude was about half that of the repression to which the construct with the full-length insert responded. Since CCAAT/enhancer-binding protein alpha (C/EBPα) [Wu et al., 1999], cAMP response element-binding protein (CREB) [Moore and Koontz, 1989], and specificity protein 1 (Sp1) [Pan et al., 2001], whose binding sites lie in the promoter region of the AQP3 gene, were reported to be involved in insulin-receptor signaling, we examined whether mRNA levels of these transcription factors were affected by insulin but found almost no change in their levels. Whether transcription factors other than Foxa2 are involved in the insulin-mediated repression of AQP3 gene expression still remains unclear and other forms of transcriptional regulations need to be investigated.

Glycogen seems to be preferred for the production of ATP since the transcriptional activity of Foxa2 was suppressed by insulin and fatty acid oxidation subsequently declined of a high enough level of blood glucose after a meal [Montminy and Koo, 2004]. Foxa2 can act as a transcriptional regulator remaining in its unphosphorylated form in the nuclear fraction when levels of insulin are low under condition of fasting. Fatty acids are believed to be burned up with an acceleration of fatty acid oxidation dependent on the transcriptional activation of Foxa2. Thus, Foxa2 seems to serve as an insulin-mediated regulator in fatty acid oxidation and glycometabolism. Our finding of insulin-mediated repression of the gene expression of AQP3 regulated by Foxa2 implies that APQ3

is involved in fatty acid oxidation and glycometabolism given its permeability to glycerol.

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