Coordinated Regulation of Fat-Specific and Liver-Specific Glycerol Channels, Aquaporin Adipose and Aquaporin 9

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Plasma glycerol is a major substrate for hepatic gluconeogenesis. Aquaporin adipose (AQPap/7), an adipose-specific glycerol channel, provides fat-derived glycerol into plasma. In the present study, we cloned the coding and promoter regions of mouse aquaporin 9 (AQP9), a liver-specific glycerol channel. Fasting and refeeding of mice increased and decreased hepatic AQP9 mRNA levels, respectively. Insulin deficiency induced by streptozotocin resulted in increased hepatic AQP9 mRNA. These changes in hepatic AQP9 mRNA were accompanied by those of hepatic gluconeogenic mRNAs and plasma glycerol levels. In cultured hepatocytes, insulin downregulated AQP9 mRNA. The AQP9 promoter contained the negative insulin response element TGTTTTC at −496/−502, similar to the promoter of the AQPap/7 gene. In contrast, in insulin-resistant *db+/db+* mice, AQPap/7 mRNA in fat and AQP9 mRNA in liver were increased, despite hyperinsulinemia, with high plasma glycerol and glucose levels. Glycerol infusion in the *db+/db+* mice augmented hepatic glucose output. Our results indicate that coordinated regulations of fat-specific AQPap/7 and liver-specific AQP9 should be crucial to determine glucose metabolism in physiology and insulin resistance. *Diabetes* 51: 2915–2921, 2002

The precise mechanism for the common association between obesity and diabetes remains unresolved. Adipose tissue supplies lipolysis-derived free fatty acid (FFA) and glycerol to other organs in various physiological conditions, such as diet restriction and physical exercise (1–4). We and others have reported that accumulation of intra-abdominal visceral fat is closely associated with increased incidence of metabolic complications in obesity (5–9). Higher influx of FFA into the liver, especially from accumulated visceral fat, drives the hepatic synthesis of triglyceride, leading to fatty liver and hypertriglyceridemia (10–12).

Glycerol, another product of lipolysis, is an important substrate for hepatic gluconeogenesis (13–15). As the substrate for hepatic glucose production, glycerol accounts for 90% in the prolonged fasting state and ~50% in the postabsorptive state in rodents (16,17). In humans, it was estimated that 20% of the gluconeogenesis was mediated by glycerol after 60 h of fasting (13,18). Higher influx of glycerol into the liver from the accumulated fat may be relevant to the development of diabetes in obesity. The molecular basis for the secretion and uptake of glycerol has been characterized recently. Aquaporins (AQPs), which are channel-forming integral proteins, function as water channels (19). To date, at least 10 AQPs have been identified (20). Functional studies have distinguished the members of the AQP family into two subgroups: AQPs that are selective water channels and aquaglyceroporins that transport glycerol as well as water (21). We recently cloned a novel adipose-specific glycerol channel, designated aquaporin adipose (AQPap/7) (22), and reported that this protein functions as a glycerol channel from fat into blood (23–25). Recently, a liver-specific aquaglyceroporin was also identified and named AQP9 (26). The significance and regulation of AQP9 in physiological and pathophysiological conditions have not been elucidated.

In the current study, we demonstrate the inhibitory effect of insulin on the expression of the AQP9 gene in normal liver through the negative insulin response element (IRE) in its promoter region (27), similar to the regulation of AQPap/7 in adipose tissue (23,24). These coordinated regulations of AQPap/7 and AQP9 should account for the physiological suppression of glycerol release from fat and gluconeogenesis in liver by feeding. We also show a dysregulated increase of AQP9 in the liver and AQPap/7 in the adipose tissue of insulin-resistant hyperinsulinemic mice. Higher amounts of glycerol, released by increased function of AQPap/7, should be taken up into hepatocytes more efficiently via enhanced AQP9, leading to augmentation of hepatic gluconeogenesis. The coordinated changes in two tissue-specific glycerol channels, adipose AQPap/7 and hepatic AQP9, may determine, at least in part, plasma glucose levels during nutritional alterations as well as in insulin-resistant conditions.

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Received for publication 15 March 2002 and accepted in revised form 26 June 2002.

AQP, aquaporin; AQPap/7, aquaporin adipose; DMEM, Dulbecco's modified Eagle's medium; FFA, free fatty acid; GlyK, glycerokinase; HSL, hormone-sensitive lipase; IRE, insulin response element; IRS, insulin receptor substrate; PEPCK, phosphoenolpyruvate carboxykinase; RACE, rapid amplification of cDNA ends; STZ, streptozotocin.

RESEARCH DESIGN AND METHODS

Materials and general methods. Plasma glycerol was measured by a fluorometric/colorimetric enzyme method (22–25). FFA was determined using Nescauto NEFA kit (Azwell, Osaka, Japan). Glucose and insulin were measured by a Blood Sugar Glucose Oxidase Perid Method kit (Roche Diagnostic, Tokyo, Japan) and a double-antibody enzyme immunoassay using a Glazyme Insulin EIA kit (Sanyo Chemical Industries, Osaka, Japan) with rat insulin as a standard, respectively.

Animals and cells. All experimental protocols described in this article were approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine. Eight-week-old male ICR (MCH), C57BL/6J, C57BL/KsJ (*db+/+m*), and C57BL/KsJ (*db+/db+*) mice were purchased from Clea Japan (Osaka, Japan). The animals were housed in an air-conditioned room with a 12/12-h dark-light cycle (8:00 A.M.–8:00 P.M.) and acclimated to the new environment for 1 week before the experiment. For experiments on insulin deficiency, streptozotocin (STZ; Sigma Aldrich, Tokyo, Japan) or PBS was administered intraperitoneally (100 mg/kg in 0.05 mol/l citrate buffer, pH 4.5) into 9-week-old male ICR (MCH) mice. Three days later, both groups of mice were anesthetized with 5 mg/ml pentobarbital sodium salt before being killed.

For experiments designed to study the effects of fasting and refeeding, male C57BL/6J mice (each group, $n = 4$) were allowed free access to standard laboratory diet and tap water for 12 h after a 24-h fast. The fasted group was fasted for 12 or 18 h before being killed. The refed group had free access to standard laboratory diet for 12 h after an 18-h fast. For comparison between the obese and lean controls, C57BL/KsJ (*db+/db+*) and C57BL/KsJ (*db+/+m*) mice on 3 h fasting were used. All mice were phlebotomized from the tail and portal vein.

A rat hepatoma H4IIE cell line (passage no. 6) was obtained from Dainippon Pharmaceutical (Osaka, Japan). H4IIE rat hepatoma cells were grown as monolayer cultures in low-glucose Dulbecco's minimal essential medium (1.0 g/l) supplemented with 10% FCS and incubated in a humidified 95% air–5% CO_2 atmosphere at 37°C until they reached preconfluence (60–70%), when they were collected for analysis (28). H4IIE hepatoma cells (preconfluent state) were washed twice with calcium- and magnesium-free PBS and incubated with Dulbecco's modified Eagle's medium (DMEM) containing 0.5% fatty acid free BSA (Sigma) for 12 h. After cells were washed twice with PBS, they were incubated in DMEM with 10 nmol/l insulin for 0, 6, or 24 h or with 0, 10, or 100 nmol/l insulin for 24 h. Total RNA was isolated and subjected to Northern blotting for AQP9.

Isolation of mouse AQP9 gene. The primer was designed to amplify the coding region of mouse AQP9, according to the sequence of rat AQP9 (AF016406) (26), which was cloned from rat. The following forward and reverseprimerswereusedforspecificPCRamplification:5′-GGCAGAAACCCCAAGATGCCTTC-3′ and 5′-CTGCAGATCTGGCCATCCCACTACATGATG-3′. RT-PCR was performed by using the template of mouse liver mRNA.

Determination of 5′ and 3′ end of mouse AQP9 by 5′ and 3′ rapid amplification of cDNA ends PCRs. For determining the 5′ and 3′ ends of mouse AQP9, the 5′ and 3′ end rapid amplifications of the cDNA ends (5′ and 3′ RACE) were conducted according to the protocol provided by the manufacturer (Marathon cDNA amplification kit; BD Biosciences Clontech Japan, Tokyo, Japan), using the following primers: first primer 5′-TTCTTCTTGGCTCGGTCCTTCTCAGAAGG*CAT*CTT-3′ (italicized translation start codon ATG), and nested primer, 5′-CTTGGGGTTTCTGCCAGAGGTAAGCTTTCCACCA-3′ for 5′ RACE; and first primer 5′-GAACCAGCCGAGAACAATCTAGAAAAACAC-3′ and nested primer 5′-AGAAAAACACGAACTCAGCGTCATCATG*TAG*-3′ (italicized translation stop codon TAG), for 3′ RACE, respectively.

Isolation of mouse AQP9 promoter. Cloning of the 5′ flanking region and promoter of the mouse AQP9 gene was performed using the GenomeWalker kit (BD Biosciences Clontech Japan) according to the protocol recommended by the manufacturer, with the following primers: primer 5′-TGCACGAGGTTCTTCTTGGCTCGGTCCTT-3′, 5′-ACGAGGTTCTTCTTGGCTCGGTCCTTCTC-3′, 5′-TTCTTCTTGGCTCGGTCCTTCTCAGAAGG*CAT*CTT-3′ (italicized translation start codon ATG).

Luciferase assay. The mouse AQP9 promoter regions (−667/−1) were amplified from mouse genomic DNA using a Mlu I site-added 5′ primer and *Xho*I site-added 3′ primers. The mouse AQP9 promoter-luciferase reporter plasmids were constructed by excising the amplified promoter fragment of AQP9 and inserting it into the *Mlu*I and *Xho*I site of the control pGL3 basic luciferase expression vector (Promega, Madison, WI). Partial deletion mutant of pGL3-AQP9 luciferase plasmid was constructed using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). IRE-deleted constructs (ΔIRE) were designed from the wild-type construct by deleting the IRE region (−502/−496). The plasmids for transfection were purified using the Endofree Plasmid kit (Qiagen, Valencia, CA).

Primary hepatocytes were isolated from nonfasting male Sprague-Dawley rats (approximate body weight 250 g), as described previously (29). Briefly, the prepared hepatocytes were attached to a 12-well plate in DMEM supplemented with 10% FCS and 100 nmol/l triiodothyronine. Four hours later, the medium was replaced with Medium 199 (M199) supplemented with 100 nmol/l dexamethasone, 100 nmol/l triiodothyronine, and 1 nmol/l insulin. For each 12-well culture plate, 50 ng of firefly (*Photinus pyralis*) luciferase plasmid, constructed from pGL3-basic luciferase expression vector and 10 ng of a sea pansy (*Renilla reniformis*) luciferase pRL-SV40 plasmid (Promega), were complexed with LipofectAMINE2000 (Life Technologies, Tokyo, Japan) following the protocol provided by the manufacturer and then used for transfection. The transfection mixture was removed 24 h after transfection, and the cells were maintained in M199 containing the indicated concentration of insulin. The cells were harvested with passive lysis buffer (Promega), and luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega) using the procedure described by the manufacturer.

Construction of cDNA probes and RNA analysis. Mouse AQPap/7, AQP9, glycerokinase (GlyK), phosphoenolpyruvate carboxykinase (PEPCK), and hormone-sensitive lipase (HSL) cDNAs were synthesized by RT-PCR as reported previously (22,30). Insulin receptor substrate (IRS)-1 or -2 cDNA probe was described previously (30). Total cellular RNA was extracted using TRIZOL reagent kit (Invitrogen, Tokyo, Japan). Northern blot analysis was performed as described previously (23). Abundance of mRNAs was determined by densitometric analysis using FAST SCAN Scanning Imager (Molecular Dynamics, Buckinghamshire, U.K.) and expressed in arbitrary units.

Immunoblot of AQPap. Immunoblot analysis of AQPap/7 protein was performed as reported previously (22). Briefly, the membrane fractions of adipose tissues were obtained. The proteins (40 μg) were loaded and separated on a 12.5% SDS–polyacrylamide gel and transferred onto nitrocellulose transfer membrane (Schleicher & Schuell, Keene, NH). Western blot analysis was performed using antiserum to AQPap/7 at a dilution of 1:500. Horseradish peroxidase–coupled donkey anti-rabbit immunoglobulins were used at a dilution of 1:3,000. Detection by chemiluminescence was performed using the ECL system (Amersham Pharmacia Biotech, Tokyo, Japan).

Mouse liver perfusion studies. Mouse liver perfusion studies were performed as described previously, with a minor modification (31,32). After 18 h of fasting, the male C57BL/KsJ (*db+/db+*) and (*db+/+m*) mice ($n = 4$, each) were anesthetized with an intraperitoneal injection of 5 mg/ml pentobarbital sodium. The abdominal cavity was opened, and the superior mesenteric artery and bilateral renal arteries and veins were ligated. Then, 0.25 ml (25 units) of heparin was injected into the inferior vena cava and the liver was perfused via the portal vein at a constant flow rate of 4 ml/min. The perfusate was albumin-free Krebs buffer (in mmol/l: NaCl 120, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, KCl 4.8, $CaCl_2 \cdot 2H_2O$ 1.72, and $NaHCO_3$) at 37°C, bubbled with 95% O_2–5% CO_2 gas mixture. The concentration of glycerol infused into the portal vein was increased every 20 min from 200 to 400, 600, and 800 μmol/l. A recovery catheter was inserted into the inferior vena cava, and samples were collected every 10 min after preperfusion for 30 min and stored at −80°C for later analysis. At the end of perfusion, the whole liver was removed and weighed. Glucose concentration in the recovery samples was measured using the Blood Sugar Glucose Oxidase Perid Method kit.

Statistical analysis. All data were expressed as mean ± SE. Differences between groups were examined for statistical significance using the Student's t test. $P < 0.05$ denoted the presence of a statistically significant difference.

RESULTS

Cloning of the mouse AQP9 gene. To investigate the regulation of AQP9, we cloned mouse AQP9 cDNA from the mouse liver. Figure 1 illustrates the amino acid alignments of AQP9 among mouse, rat, and human. The mouse AQP9 cDNA encodes a 295 amino acid (GenBank accession no. AB 037180) and shows 90% homology to rat AQP9 (GenBank accession no. AF 016406) (26) and 76% homology to human AQP9 (GenBank accession no. XM 012421) (33,34). Hydropathy analysis showed that mouse AQP9 has six putative transmembrane domains, similar to human and rat AQP9. Mouse AQP9 has two Asn-Pro-Ala consensus motifs, characteristic of members of the major intrinsic protein family (20).

Effect of fasting/refeeding on AQP9, GlyK, and PEPCK mRNA expressions. Fasting activates lipolysis and glycerol release from adipose tissue (13). We recently

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                1                                  30
m AQP9  MPSEKDRAKKNLVQRLALKSCLAKETLSEF
r AQP9  MPSEKDGAKKSLMQRLALKSRIAKETLSEF
h AQP9  MQPEGAEKGKSFKQRLVLKSSLAKETLSEF

m AQP9  LGTFIMIVLGCGSIAQAVLSREKAGGIITI
r AQP9  LGTFIMIVLGCSSIAQAVLSRERFGGIITI
h AQP9  LGTFILIVLGCGCVAQAILSRGRFGGVITI
                    ①
m AQP9  NIGFATAVVMALYATFGVSGGHINPAVSFA
r AQP9  NIGFASAVVMALYVTFGISGGHINPAVSFA
h AQP9  NVGFSMAVAMAIYVAGGVSGGHINPAVSLA
                    ②
m AQP9  MCTFGRMEWFKFPFYVGAQLLGAFVGAATV
r AQP9  MCAFGRMEWFKFPFYVGAQFLGAFVGAATV
h AQP9  MCLFGRMKWFKLPFYVGAQFLGAFVGAATV

m AQP9  FGIYYDGLMAFADGKLLITGENGTAFIFAT
r AQP9  FGIYYDGLMAFAGGKLLVVGENATAFIFAT
h AQP9  FGIYYDGLMSFAGGKLLIVGENATAHIFAT
                  ③
m AQP9  YPKPFVSVPGAFVDQVVSTMFLLLIVFAIF
r AQP9  YPAPFISTPGAFVDQVVSTMFLLLIVFAMF
h AQP9  YPAPYLSLANAFADQVVATMILLIIVFAIF
                          ④
m AQP9  DSRNLGVPRGLEPIVIGLLIIVISCSLGLN
r AQP9  DSRNLGVPRGLEPVVIGLLIIVLSCSLGLN
h AQP9  DSRNLGAPRGLEPIAIGLLIIVIASSLGLN
                          ⑤
m AQP9  SGCAMNPARDLSPRLFTALAGWGFEVFTFG
r AQP9  SGCAMNPARDLSPRLFTALAGWGFEVFTVG
h AQP9  SGCAMNPARDLSPRLFTALAGWGFEVFRAG

m AQP9  NNFWWIPVVGPMIGAVLGGLIYVLFIQMHH
r AQP9  NNFWWIPVVGPMIGAFLGGLIYILFIQMHH
h AQP9  NNFWWIPVVGPLVGAVIGGLIYVLVIEIHH
                          ⑥          295
m AQP9  SNPDPEVKAEPAENNLEKHELSVIM
r AQP9  SKLDPDMKAEPSENNLEKHELSVIM
h AQP9  PEPDSVFKTEQSEDKPEKYELSVIM
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FIG. 1. Deduced amino acid sequence of mouse AQP9. Amino acid sequence alignments of mouse AQP9 (m; GenBank accession no. AB 037180) isolated from the mouse liver, rat AQP9 (r; GenBank accession no. AF 016406) (24), and human AQP9 (h; GenBank accession no. XM 012421) (31). Asn-Pro-Ala (NPA) motifs are boxed, and putative membrane-spanning regions are underlined and numbered 1–6 inside a circle. The conserved residues are indicated by bold letters.

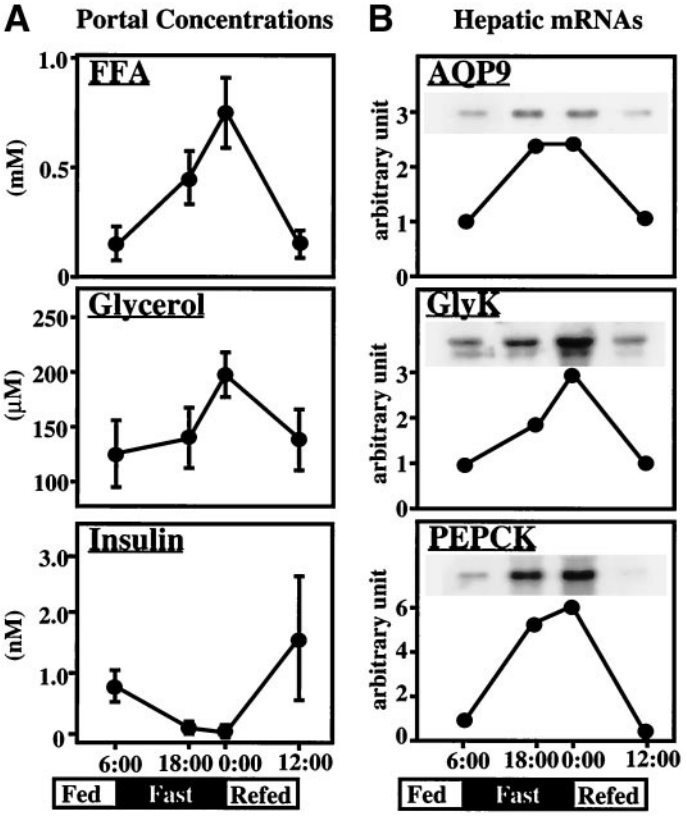

FIG. 2. Effect of fasting/refeeding on expression of AQP9, GlyK, and PEPCK mRNAs. Mice were killed after 0, 12, and 18 h of fasting or 12 h of refeeding after 18 h of fasting and then the livers were removed for analysis. *A*: Plasma FFA, glycerol, and insulin in the portal vein were measured and the data are mean ± SE. *B*: Northern blots using pooled RNA samples (10 μg/lane). Abundance of mRNAs was determined by densitometric analysis using FAST SCAN Scanning Imager (Molecular Dynamics, Buckinghamshire, U.K.) and is presented in arbitrary units. Autoradiographs of the blot were inserted.

reported that AQPap mRNA expression in white adipose tissue was enhanced by fasting and suppressed by refeeding and that the changes in AQPap mRNA showed a pattern similar to that of plasma glycerol (23). Glycerol becomes a substrate for gluconeogenesis in the liver that expresses GlyK (35). Figure 2 shows the effects of fasting and refeeding on the regulation of hepatic mRNAs of AQP9 and GlyK. Portal plasma levels of glycerol and FFA increased during fasting and decreased after refeeding (Fig. 2*A*). In contrast, portal plasma insulin levels decreased during fasting and increased after refeeding (Fig. 2*A*). The mRNA levels of AQP9 and GlyK increased significantly by fasting and decreased by refeeding, similar to the change in PEPCK mRNA (Fig. 2*B*) (36), which is known to be downregulated by insulin. The coordinated enhancements of AQPs (22), GlyK, and PEPCK are effective to supply plasma glycerol for hepatic gluconeogenesis in fasting. Similar effects of fasting on these hepatic mRNAs were also seen when fasting started at 0:00 h.

Effect of insulin on AQP9 mRNA expression in mouse liver and H4IIE cells. Next, we examined the regulation of hepatic AQP9 mRNA by insulin (Fig. 3). Hepatic mRNA levels of AQP9 and GlyK were high in insulin-deficient STZ-treated mice, similar to that of PEPCK mRNA (Fig. 3*A*). Furthermore, insulin downregulated the expression levels of AQP9 mRNA in H4IIE hepatoma cells in dose- and time-dependent manners (Fig. 3*B*). These results suggested that the AQP9 gene expression is directly downregulated by insulin, similar to PEPCK mRNA.

Promoter of mouse AQP9 gene. The 5′ flanking region of the mouse AQP9 gene was sequenced (GenBank accession no. AB 073723), and transcriptional initiation site of the AQPap/7 gene was determined by 5′ RACE using total RNA isolated from mouse liver (Fig. 4*A*). A search of the promoter region of the AQP9 gene for canonical consensus sequences revealed the presence of several putative binding sites for transcription factors (Fig. 4*A*). Several binding sites for CCAAT enhancer binding protein and hepatic nuclear factor-3β were identified in the promoter (23).

Negative IRE in the mouse AQP9 gene promoter. In the mouse AQP9 gene promoter, we identified a region similar to the core negative IRE, T(G/A)TTTT(G/T) (27,37), which is also found in promoters of various genes, including PEPCK (38) and AQPap/7 (23,24) (Fig. 4*B*). For

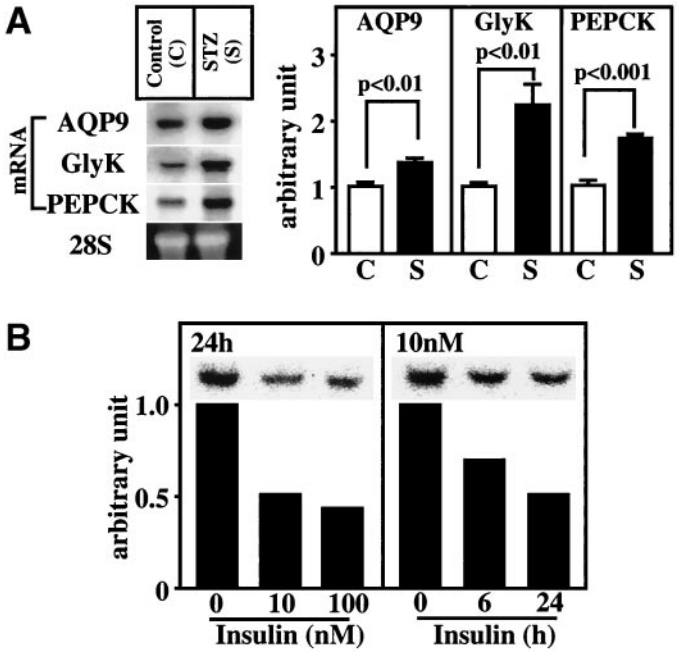

FIG. 3. Effect of insulin on AQP9 mRNA expression in mouse liver and H4IIE cells. *A*: Expression of AQP9, GlyK, and PEPCK mRNAs in livers of insulin-deficient mice. Insulin-deficient state was generated by streptozotocin (STZ) treatment as described in RESEARCH DESIGN AND METHODS. Total RNA (10 μg/lane) of the liver from each mouse in control (C, $n = 4$) and STZ-treated (S, $n = 4$) mice were subjected to Northern blotting, and the results were quantified, as described in the legend to Fig. 2*B*. A representative autoradiograph shows AQP9, GlyK, and PEPCK mRNA bands and a photograph of the same gel after ethidium bromide staining (showing 28S ribosomal RNA, below; left). *B*: Rat H4IIE hepatoma cells were preincubated with DMEM containing 0.5% BSA for 12 h. After washing, the cells were incubated with DMEM containing 0.5% BSA and the indicated concentration of insulin for 24 h or 10 nmol/l insulin for 0, 6, or 24 h. RNAs samples (10 μg/lane) were subjected to Northern blot analysis. Quantification of the results was performed as described in the legend to Fig. 2*B*. Autoradiographs of the blot were inserted.

determining whether the putative IRE is a specific region required for insulin-mediated repression of AQPap transcription, deletion mutant of the IRE in the mouse AQP9 promoter was subcloned into luciferase vectors (Fig. 4*C*). The wild-type construct containing the native −667/−1 regions showed 45% inhibition of luciferase activity after treatment with insulin (Fig. 4*C*). In contrast, the construct lacking IRE was completely resistant to the inhibitory effect of insulin on promoter activities as a result of the reduced basal promoter activity. Insulin suppressed the wild-type luciferase activity in rat primary hepatocytes in a dose-dependent manner (Fig. 4*D*). In the absence of insulin, the wild-type AQP9 promoter produced a higher luciferase activity than the deletion mutant (ΔIRE) promoter. In the presence of insulin, the activity of the wild-type AQP9 promoter was reduced to the level of the mutant promoter, which was not affected by insulin. Next, we examine the point mutation analysis (Fig. 4*E*). The activity of the wild-type AQPap/7 promoter was reduced to 55% in the presence of insulin, similar to the results in Fig. 4*C*. Each mutation in base pair 2 (G→C) and 3 (T→A) of the heptanucleotide sequence completely blocked insulin-sensitive repression of mouse AQP9 transcription (Fig. 4*E*), similar to the results of mouse and human AQPap/7 promoters that we recently reported (23,24). These results confirm that the IRE sequence (−502/−496) is required for

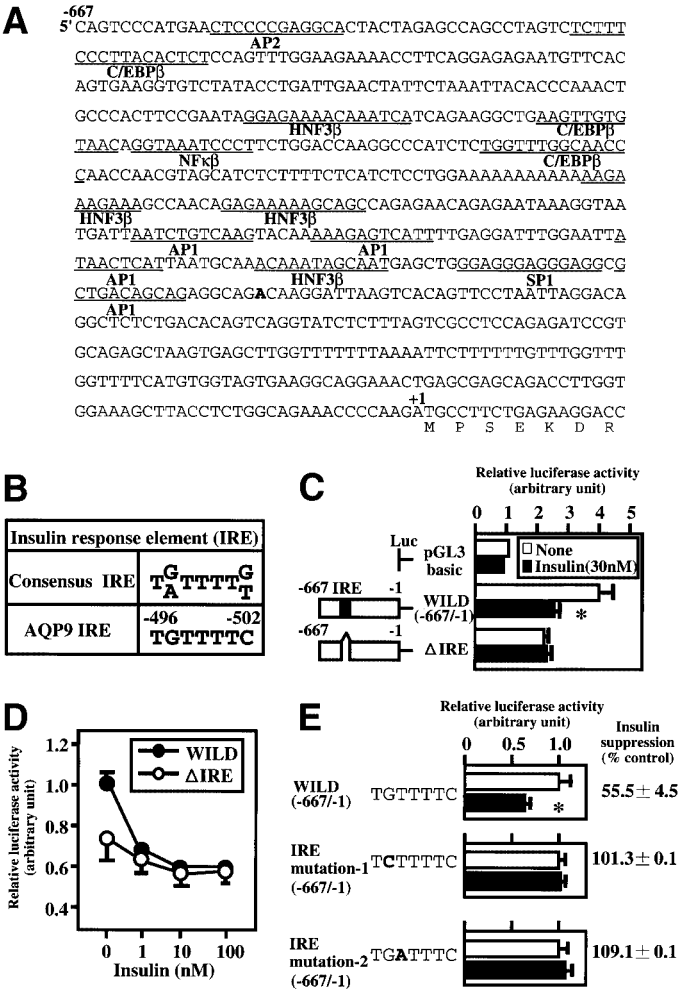

FIG. 4. Insulin-mediated suppression of mouse AQP9 gene transcription through the IRE. *A*: Promoter sequence of mouse AQP9 gene. Sequence of the mouse AQP9 promoter and its 5′-flanking sequence (GenBank accession no. AB 073723). The translation initiation site of AQP9 is designated +1. Transcription start site predicted by 5′-RACE as human AQP9 gene (27) is shown in bold. Putative transcription factor binding sites are predicted by the sequence motif search program, MatInspector V2.2 (transfac.gbf.de/cgibin/matSearch/matsearch.pl). One reverse-oriented IRE is boxed with a solid line. *B*: Consensus sequence of IRE and the putative IRE of the AQP9 gene (−496/−502). *C*: Schematic presentation of the plasmid constructs used to identify the insulin response sequence in the promoter of the human AQP9 gene. Primary rat hepatocytes were cotransfected with pRL-SV40 plasmid and the indicated constructs and incubated in the presence (■) or absence (□) of insulin as described in RESEARCH DESIGN AND METHODS. Twelve hours later, cells were harvested for the measurement of luciferase activity. The value for non–insulin-treated pGL3-basic luciferase activity was arbitrarily set as 1.0. Normalized luciferase activities are expressed as mean ± SE ($n = 3$). *$P < 0.05$ (Student's t test), between the control group and insulin-treated group. *D*: Dose curve of insulin-mediated inhibition of AQPap promoter activity in rat primary hepatocytes. Primary hepatocytes were cotransfected with pRL-SV40 plasmids and either pAQP9 (WILD)-luciferase (●) or pAQP9 (ΔIRE)-luciferase (○) for 24 h and then incubated for 12 h with the indicated concentration of insulin. Cells were harvested for measurement of luciferase activity. The value for pAQP9 (WILD)-luciferase activity in the absence of insulin was arbitrarily set as 1.0. Data are expressed as mean ± SE ($n = 3$). *E*: Primary rat hepatocytes were cotransfected with pRL-SV40 plasmid and the wild-type or the mutant plasmids in which the indicated single base pair was substituted in the IRE sequence and incubated in the presence (■) or absence (□) of insulin, as described in RESEARCH DESIGN AND METHODS. Twelve hours later, cells were harvested for the measurement of luciferase activity. The value for non–insulin-treated pGL3-basic luciferase activity was arbitrarily set as 1.0. Normalized luciferase activities are expressed as mean ± SE ($n = 4$). *$P < 0.05$ (Student's t test), between the control group and insulin-treated group. This experiment was performed three times with similar results.

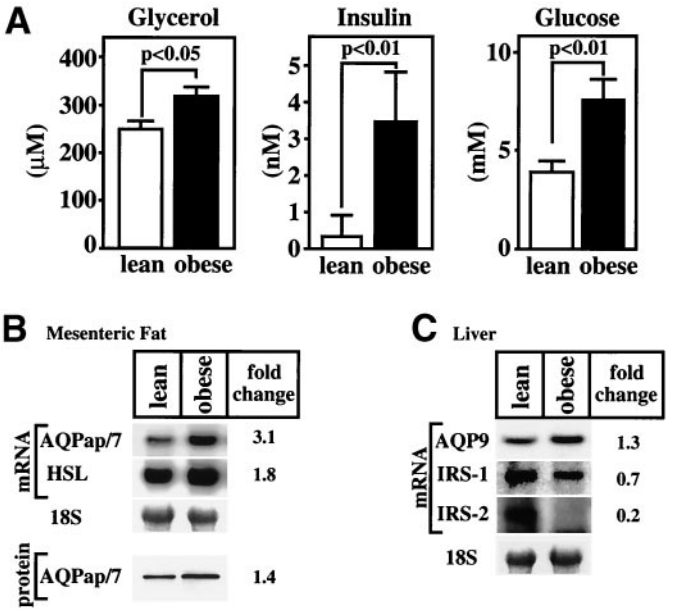

FIG. 5. Metabolic profiles and AQPap/7 and AQP9 mRNA expression in *db+/db+* mice. *A*: Plasma concentrations of insulin, glucose, and glycerol in *db+/+m* (lean) and *db+/db+* (obese) mice (n = 4, each) after 3 h of fasting. Data are mean ± SE, and comparisons are between lean and obese (Student's t test). *B*: Northern blotting of AQPap/7 and HSL and Western blotting of AQPap/7 from the mesenteric fat of *db+/+m* and *db+/db+* mice. Northern and Western blotting were performed as described in RESEARCH DESIGN AND METHODS. *C*: Northern blotting of AQP9, IRS-1, and IRS-2 from the livers of *db+/+m* and *db+/db+* mice. For *B* and *C*, the fold changes in obese mice, compared with lean mice, were calculated from the results of individual animals in each group.

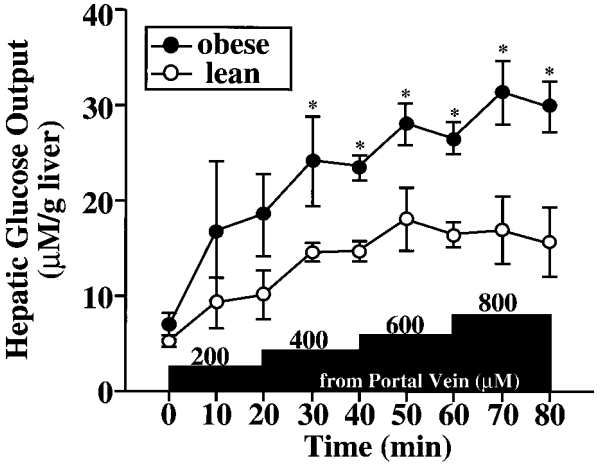

FIG. 6. Hepatic glucose production from glycerol in control and *db+/db+* mice. Hepatic glucose production from glycerol in *db+/db+* (obese) and *db+/+m* (lean) mice (n = 4, each) after 18 h of overnight fasting was measured using the hepatic perfusion procedure as described in RESEARCH DESIGN AND METHODS. Glycerol was infused from the portal vein at the indicated concentration, and the concentration was increased every 20 min. The recovery samples were collected from the inferior vena cava every 10 min, and glucose concentration was measured. Data are mean ± SE; *P < 0.05, between lean and obese mice (Student's t test).

insulin-mediated suppression on the transcription of the mouse AQP9 gene.

Metabolic profile and expression of AQPap/7 and AQP9 mRNAs in *db+/db+* mice. The *db+/db+* mice were significantly insulin resistant. Plasma glycerol levels in the portal vein were higher in *db+/db+* mice. They had a higher concentration of plasma glucose despite severe hyperinsulinemia (Fig. 5*A*). Figure 5*B* and *C* demonstrates the results of Northern blotting of mesenteric fat and liver, respectively. The mRNA and protein amounts of AQPap/7 and HSL mRNA were higher in the mesenteric fat of *db+/db+* mice (Fig. 5*B*), although both are negatively regulated by insulin. The hepatic AQP9 mRNA was also induced despite hyperinsulinemia (Fig. 5*C*). Hepatic insulin resistance was accompanied by markedly reduced IRS-2 (Fig. 5*C*), as reported previously (30).

Hepatic glucose production from glycerol in control and *db+/db+* mice. Glycerol is the main gluconeogenic substrate in prolonged fasting (15–17). Hepatic glucose production from glycerol was compared between db+/db+ and *db+/+m* mice in the 18-h fasted state using a previously described hepatic perfusion procedure (31,32). Glucose production increased in parallel with the amount of glycerol perfused into the portal vein in *db+/+m* and *db+/db+* mice (Fig. 6). Glucose output was significantly higher in the liver of *db+/db+* mice than *db+/+m* mice. These results indicated that the glycerol-mediated gluconeogenetic activity was augmented in the liver of *db+/db+* mice (39), where the mRNAs of AQP9 and gluconeogenic enzymes were augmented.

DISCUSSION

Glycerol is one of the major factors that determine plasma glucose (40). During fasting, hepatic glucose output is the main source of plasma glucose, and glycerol taken up from plasma becomes a major substrate for hepatic gluconeogenesis (14). However, adipose tissue is the major source of plasma glycerol (13). Therefore, effective systems are required during fasting that enhance glycerol release from adipose tissue and glycerol uptake into the liver. With regard to the glycerol transport proteins, three aquaglyceroporins are known: AQPap/7, AQP3, and AQP9 (20,22). To date, AQPap/7 is the only aquaglyceroporin expressed in adipose tissues, and AQP9 is the only aquaglyceroporin expressed in the liver (22). It is conceivable that during fasting, AQPap/7 in fat is essential for the supply of glycerol to the liver and that AQP9 is critical for hepatic uptake of glycerol for glucose production.

Insulin suppresses the amount of AQPap/7 mRNA in adipose tissue (23) and AQP9 in liver (current study). We also identified similar negative IREs in the promoter regions of AQPap/7 (23,24) and AQP9 genes (current study). We identified the potential IRE sequence (−591/−597; TGTTTTC) in the promoter region of the human AQP9 gene (33). These results indicate that the gene expressions of AQPap/7 and AQP9 are regulated coordinately by the plasma concentrations of insulin in accordance with the nutritional condition, such as fasting and refeeding. These regulations should increase or decrease the supply of glycerol and glucose in plasma by fasting or refeeding, respectively.

In hepatic gluconeogenesis, AQP9, which is localized at the sinusoidal plasma membrane (41), serves as a channel for glycerol uptake, GlyK converts the incorporated glycerol into the phosphorylated form to be used for gluconeogenesis, and PEPCK is one of the limiting enzymes to drive the glycerol-independent gluconeogenic pathway (2). In the normal liver, the mRNAs for all of these proteins

are downregulated by insulin (27,37). During fasting, the low concentration of plasma insulin coordinately and effectively enhances all of these mRNAs to drive hepatic gluconeogenesis. However, under pathological conditions of insulin resistance in the *db/db* mice, high mRNA expression levels of hepatic AQP9, GlyK, and PEPCK mRNAs and of adipose AQPap/7 were found despite the high concentration of plasma insulin. Dysregulated increase of AQPap/7 and hepatic AQP9 may underlie the pathophysiology of hyperglycemia in severe insulin resistance. Our recent studies indicated that the dysregulated increase of AQPap/7 in adipose tissues causes higher plasma glycerol in insulin-resistant rodents (22). The liver of insulin-resistant *db+/db+* mice exhibited a high gluconeogenic activity by using glycerol as a substrate (Fig. 6). Hepatic insulin resistance may be accounted for by the significantly low levels of IRS-2. We previously demonstrated that chronic hyperinsulinemia in lipodystrophy and *ob/ob* mice was associated with low hepatic IRS-2, which resulted in increased mRNAs of gluconeogenesis, such as glucose-6-phosphatase and PEPCK (30). There were no apparent changes in adipose mRNAs for IRS-1 and IRS-2 between obese and lean mice (data not shown). In the previous articles, IRS-1–mediated insulin signaling was shown to be deteriorated in the adipose tissue of insulin-resistant animals, with relatively normal IRS-2–mediated pathway (42,43). We previously showed that insulin's inhibition of AQPap/7 was mediated by a phosphatidylinositol 3-kinase–dependent manner (23). Taken together, the increase of AQPap under insulin resistance may be caused by the decreased IRS-1–mediated insulin signaling in the adipose cells. In the present study, it is conceivable that reduced hepatic IRS-2 in *db+/db+* mice resulted in disturbance of insulin signaling, which led to an increase in AQP9 and hepatic glycerol uptake. In the insulin-resistant state, both the level of plasma glycerol and the glucose production associated with glycerol input were increased, which might lead to the more severe hyperglycemia. Coordinated augmentation of AQPap/7 and AQP9 in insulin resistance should increase the supply of fat-derived glycerol as a substrate for hepatic gluconeogenesis, which aggravates hyperglycemia.

Figure 7 represents a summary of the working model based on the results of our previous (21–25) and present studies. In the physiological feeding state, high plasma insulin coordinately suppresses the AQPap/7 mRNA for glycerol release from fat and AQP9 mRNA for glycerol uptake into the liver, through the negative IRE in AQPap/7 and AQP9 gene promoters. In contrast, in the insulin-resistant state, reciprocal increases of AQPap/7 and AQP9 despite hyperinsulinemia lead to more utilization of the fat-derived glycerol for hepatic glucose production and release. Identifying the responsible transcriptional factors and associated proteins involved in the transcriptional regulation of AQPap/7 and AQP9 genes and defining the precise mechanism that modifies the amounts and activities of these proteins by insulin should provide pivotal insight for the design of new pharmaceutical strategies to combat insulin resistance syndrome.

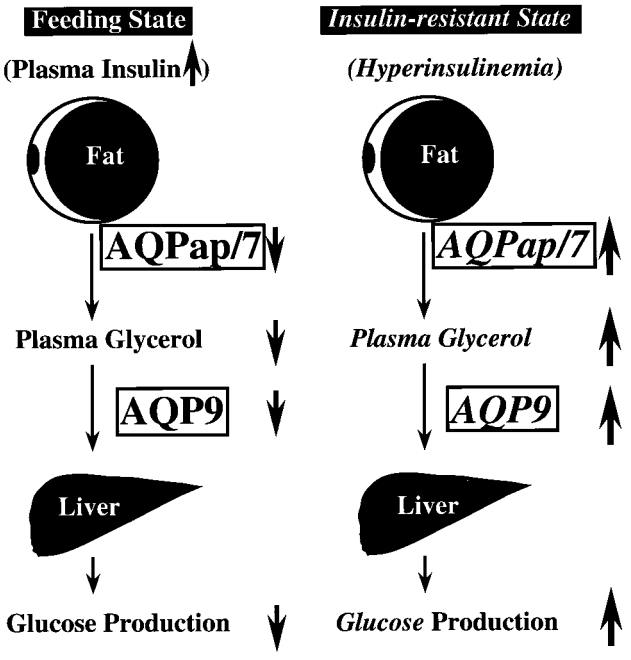

FIG. 7. Physiological and pathophysiological involvement of AQPap/7 and AQP9. In the physiological feeding state, high plasma insulin suppresses AQPap/7 mRNA in the fat and AQP9 mRNA in the liver through the negative IRE in AQPap/7 and AQP9 gene promoters. These coordinated regulations efficiently reduce the supply of glycerol from fat to liver for hepatic glucose production. In contrast, in the pathological state of insulin resistance, increased AQPap/7 and AQP9 mRNAs, despite hyperinsulinemia, augment the utilization of glycerol for hepatic glucose production. These coordinated dysregulations of AQPap/7 and AQP9 aggravate the hyperglycemia in the insulin-resistant state.

ACKNOWLEDGMENTS

This work was supported in part by the fund from the "Research for the Future" Program from the Japan Society for the Promotion of Science (JSPS-RFTF97L00801) and Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (09307019, 10557100, 10557101, 10671035, and 13671189).

We thank Yuko Matsukawa and Sachiyo Tanaka of our laboratory for technical assistance.

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