Aquaporin Adipose, a Putative Glycerol Channel in Adipocytes*

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Adipose tissue is a major site of glycerol production in response to energy balance. However, molecular basis of glycerol release from adipocytes has not yet been elucidated. We recently cloned a novel member of the aquaporin family, aquaporin adipose (AQPap), which has glycerol permeability. The current study was designed to examine the hypothesis that AQPap serves as a glycerol channel in adipocytes. Adipose tissue expressed AQPap mRNA in high abundance, but not the mRNAs for the other aquaglyceroporins, AQP3 and AQP9, indicating that AQPap is the only known aquaglyceroporin expressed in adipose tissue. Glycerol release from 3T3-L1 cells was increased during differentiation in parallel with AQPap mRNA levels and suppressed by mercury ion, which inhibits the function of AQPs, supporting AQPap functions as a glycerol channel in adipocytes. Fasting increased and refeeding suppressed adipose AQPap mRNA levels in accordance with plasma glycerol levels and oppositely to plasma insulin levels in mice. Insulin dose-dependently suppressed AQPap mRNA expression in 3T3-L1 cells. AQPap mRNA levels and adipose glycerol concentrations measured by the microdialysis technique were increased in obese mice with insulin resistance. Accordingly, negative regulation of AQPap expression by insulin was impaired in the insulin-resistant state. Exposure of epinephrine translocated AQPap protein from perinuclear cytoplasm to the plasma membrane in 3T3-L1 adipocytes. These results strongly suggest that AQPap plays an important role in glycerol release from adipocytes.

Aquaporins (AQPs),† which are channel-forming integral proteins, function as water channels (1). To date, at least 10 AQPs have been identified and cloned in mammalian tissues actively transporting water molecules, including AQP0 in lens fiber cells (2), AQP1 in erythrocytes (3), AQP2 (4), AQP3 (5, 6), and AQP6 (7) in the kidney, AQP4 in the brain (8), AQP5 in lacrimal and salivary glands (9), AQP7 in the testis (10), AQP8 in the pancreas (11) and testis (12), and AQP9 in the liver (13). These AQPs regulate water movement and are involved in the correction of the osmotic pressure gradient. The fact that mutations in the AQP2 gene cause nephrogenic diabetes insipidus in humans has emphasized the importance of the molecule on regulating water balance (14). Previously, we isolated a novel cDNA belonging to the AQP family from the human adipose tissue cDNA library and revealed that its mRNA was expressed abundantly in human adipose tissue (15). Therefore, we named it aquaporin adipose (AQPap).

Functional studies have distinguished the members of AQP family into two subgroups: aquaporins which are selective water channels, and aquaglyceroporins which transport glycerol as well as water (16). In our previous study (15), injection of AQPap cRNA into Xenopus oocytes exhibited permeability to glycerol as well as water, indicating that AQPap belongs to the aquaglyceroporin group.

Glycerol is one of the essential nutrients in the mammalian body. In fasting, triglyceride in adipocytes is hydrolyzed to glycerol and free fatty acid (FFA) by hormone-sensitive lipase (HSL) and both are released into the bloodstream. Organs expressing glycerokinase, such as the liver and kidney, take up glycerol and use it for gluconeogenesis to maintain plasma glucose levels (17, 18). It had been believed that transport of liberated fatty acids from adipocytes was achieved by simple diffusion (19). However, recent studies have identified several membrane proteins that transport FFA in adipocytes. These include fatty acid transport protein (FATP) (19, 20), plasma membrane fatty acid-binding protein (21), and fatty acid translocase (19, 22). In contrast, the molecular mechanism underlying glycerol transport across the cell membrane has not yet been elucidated. A specific molecule(s) is thought to participate in facilitating glycerol transport from the cell similar to FFA transport. AQPap mRNA is expressed predominantly in adipose tissue in humans. In the current study, we examined the regulation of AQPap in both animals and tissue culture. The results strongly suggest that AQPap is the physiological glycerol channel specific to adipocytes.

EXPERIMENTAL PROCEDURES

Animals and Cells—Eight-week-old male C57BL/6J, C57BL/KsJ (db+/+), and C57BL/KsJ (db/db) mice were purchased from Clea Japan, Inc. The animals were kept at a constant room temperature with a 12 h dark-light cycle (8 a.m.–8 p.m.). They were acclimated to the new environment for 1 week before the experiment. A mouse 3T3-L1 cell line was purchased from Health Science Research Resources Bank (Osaka, Japan).
Japan, cell number JCRB9014). Cells were maintained and differentiated with 5 μg/ml insulin, 0.5 mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone according to the modified method of Rubin et al. (23).

Cloning of Mouse cDNA Probes and RNA Analysis—Mouse AQAP, AQP3, AQP9, HSL, and perilipin (derived from sequences common to both A and B domains) were synthesized by the reverse transcription-polymerase chain reaction using mouse fat tissue (AQAP, HSL, and perilipin) (24), kidney (AQP3) and liver (AQP9) RNAs as templates and used as probes for Northern blot analysis. The nucleotide sequence of mouse AQP3 and AQP9 has been deposited in DDBJ under accession numbers AB019809 and AB037180, respectively. Mouse β-actin cDNA was purchased from Stratagene Ltd. Total cellular RNA was prepared with TRIZOL® reagent kit (Life Technologies, Inc.). Northern blot analysis and RNase protection assay were performed as described previously (25, 26).

Expression of AQPs mRNAs in Mouse Tissues—Tissue distribution of AQAP, AQP3, and AQP9 mRNAs was examined in 9-week-old male C57BL/6J mice. Overnight fasted mice were anesthetized by 5 mg/ml pentobarbital sodium salt and killed. Various tissues were removed and used for RNA isolation. The pooled RNA samples from each tissue were used for Northern analysis. Effect of fasting and feeding on AQAP mRNA expression in adipose tissue was examined in male C57BL/6KsJ (db/db) mice (n = 8, each). The fed group was given free access to standard laboratory chow and the fasted group was fasted for 18 h before time point. For the experiment on the time course of fasting and refeeding, male C57BL/6J mice (each group, n = 3) were given free access to standard laboratory chow and tap water for 12 h after 24 h fasting to strictly determine the starting point of fasting. Actual fasting was started at 6 a.m. (the zero time). The fasted group was fasted for 12 (6 p.m.) or 18 (0 a.m.) hours before killing. The refed group was given free access to standard laboratory chow for 12 h after 18 h fasting. All mice were phlebotomized from the vena cava quickly. Plasma glycerol, FFA, and insulin were measured by the UV method (Roche Molecular Biochemicals, sensitivity > 1 μM), Nescauto NEFA kit (Axeal, Japan), and a double-antibody sandwich enzyme immunoassay using a Glucyl Insulin ELISA Kit (Sanyo Chemical Industries, Ltd., Japan) with rat insulin as a standard, respectively. Subcutaneous fat tissues were removed and used for RNA isolation. AQAP mRNA expression in adipose tissue of obese C57BL/6KsJ (db/db) mice was compared with that of their lean littermate C57BL/6KsJ (db+) mice (n = 8, each).

Measurement of Glycerol Concentration in Subcutaneous Fat Tissue—Glycerol concentration in subcutaneous fat tissues was measured by a microdialysis technique (27, 28). C57BL/6KsJ (db+/+db+) and (db+/+db+) mice (n = 7, each) were brought to the laboratory at 8 a.m. under ad libitum feeding and drinking condition. During the experiments, the mice were anesthetized and warmed with heating pads to maintain the body temperature at about 35-37° C. The microdialysis probes were hand-made (10 × 0.2 mm, 50,000 molecular weight cut-off). The recovery rate in the probes was calculated by in vitro experiments prior to the study. During dialysis, the probe and catheter were connected to a microinfusion pump (CMA102, BAS) and perfused with Krebs-Ringer solution. The dialysis probe was inserted into the inguinal subcutaneous fat tissue and perfusion with Krebs-Ringer solution (2 μl/min) was started. The blood samples were drawn from the jugular vein. Fractions of dialysate were collected every 30 min. The first and second fractions were discarded because of a transient rise in the concentration of glycerol in response to the initial trauma. The in vitro recovery rate of glycerol was approximately 70%. Glycerol concentration in the perfusate was measured by a fluorometric/colorimetric enzyme method (29).

Glycerol Release from 3T3-L1 Cells—3T3-L1 cells on day 9 after differentiation were washed twice with phosphate-buffered saline (PBS) without calcium and magnesium ions, and incubated with Dulbecco’s modified Eagle’s medium (DMEM) containing 0.5% fatty acid-free bovine serum albumin (BSA) (Sigma) supplemented with and without 10^{-6} M epinephrine for 1 h at 37° C (30). Glycerol concentration in the media was measured by the UV method. The effect of mercury ions on glycerol release was tested as follows. 3T3-L1 cells on day 9 after differentiation were washed twice with PBS followed by incubation with PBS containing various concentration of HgCl_{2} for 5 min. After incubation, the cells were washed and further incubated with or without 10 μM 2-mercaptoethanol for 30 min at 37° C. After washing twice with PBS, the cells were incubated with PBS containing 0.5% BSA and 10^{-6} M epinephrine for 1 h at 37° C. The concentration of glycerol in the incubated buffer was measured as described above.

Effects of Hormones on AQAP Expression in 3T3-L1 Cells—3T3-L1 cells on day 9 were used for the experiments on the effects of hormones on AQAP expression (30). Cells were washed twice with PBS and incubated with DMEM containing 0.5% BSA for 12 h. After washing twice with PBS, the cells were incubated in DMEM containing 0.5% BSA supplemented with or without 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M bovine insulin for 6 h. For the experiment on time course, the cells were incubated in DMEM with 10^{-6} M insulin for 0, 3, or 6 h. Total RNA was isolated and used for Northern blotting and RNase protection assay.

Generation of Anti-peptide Antibodies and Immunocytochemical Detection of AQAP—Polyclonal rabbit antisera was raised against the carboxyl terminal region of mouse AQAP. We designed two synthetic peptides containing YLGLIHPSIPQDPQKRLENF and NPTARDQKVTRAS (with NH_{2}-terminal cystein added in the latter synthetic amino acid peptide) because they were divergent among the AQP family. The specificity of antisera was confirmed by immunoblotting. For immunoblotting, crude membrane fractions of 3T3-L1 cells and mouse adipose tissues were prepared as follows. For 3T3-L1 cells, the cells were homogenized in buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 255 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin, then centrifuged at 8,000 x g for 10 min at 4° C. The supernatant was recentrifuged at 100,000 x g for 1 h. The crude membranes and a supernatant containing cytosol for adipose tissues, the membrane fractions were obtained by the method of Oka et al. (31, 32). The proteins (40 μg) from each fraction were loaded and separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membrane. Western blot analysis was performed using antisera to AQAP at a dilution of 1:500. Horseradish peroxidase-coupled donkey anti-rabbit immunoglobulins were used at a dilution of 1:1,000. Detection by chemiluminescence was performed using ECL™ system (Amersham Pharmacia Biotech).

For immunocytochemical detection, 3T3-L1 adipocytes were differentiated on glass coverslips and analyzed on day 13 after differentiation. Cells were incubated with or without 10^{-6} M epinephrine for 20 min at 37° C. After washing with PBS, cells were fixed with 4% paraformaldehyde for 30 min on ice, permeabilized with 0.1% Triton X-100 for 5 min on ice, washed extensively with PBS, blocked with 5% normal swine serum for 30 min, and incubated with antiseraum to AQAP serum at a dilution of 1:500 in PBS containing 5% BSA for 1 h at room temperature. Cells were washed with PBS and incubated with biotinylated swine serum to anti-rabbit immunoglobulins in PBS containing 5% BSA for 30 min at room temperature. After washing with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated avidin D for 30 min at room temperature. After extensive washing with PBS, the cells were examined by confocal microscopy and photographed.

Statistical Analysis—The results were expressed as mean ± S.E. The significance of the difference between the mean values of the groups was evaluated by Student’s t test or analysis of variance (ANOVA) with Fisher’s LSD test.
RESULTS

Tissue Distribution of Aquaglyceroporins mRNAs in Mice—

Tissue distribution of AQPap was examined in pooled RNA samples of overnight fasted mice by Northern blotting. Mouse AQPap mRNA was expressed predominantly in white and brown adipose tissues (Fig. 1). Much fainter signals were observed in kidney and skeletal (gastrocunemius) muscle. The expression pattern was similar to that in human tissues as previously reported (15). We also examined the expression of mRNAs for AQP3 and AQP9, which are other members of aquaglyceroporins shown to have the ability to transport glycerol. The results of Northern blotting showed that white and brown adipose tissues did not have any detectable amounts of AQP3 and AQP9 mRNAs (Fig. 1). Therefore, AQPap is the sole aquaglyceroporin among the known AQPs.

Effect of Fasting and Refeeding on AQPap mRNA Expression—Fasting activates lipolysis and accelerates glycerol release from adipose tissue. We investigated the effect of fasting on AQPap mRNA expression in white adipose tissues. Mice fasted for 18 h exhibited remarkably higher levels of adipose AQPap mRNA compared with the fed mice (Fig. 2a). The time course of AQPap mRNA levels during fasting and refeeding were examined in white adipose tissue of C57BL/6J mice. Mice were killed after 0, 12, and 18 h fasting or after 12 h refeeding, then white adipose tissues were removed for analysis of AQPap and HSL mRNA. Abundance of mRNAs was determined by densitometric analysis and represented by arbitrary units. Plasma glycerol, FFA, and insulin were measured and the data were represented by the mean ± S.E.

Glycerol Concentration and AQPap mRNA Levels in Adipose Tissues of Obese Mice—Glycerol metabolism is dysregulated in obesity with insulin resistance (33). Plasma glycerol concentration is elevated in obesity and non-insulin-dependent diabetes mellitus. We compared metabolic parameters in obese db+/- db+/- mice (n = 8) and lean db+/- db+/- mice (n = 7) were allowed to feed ad libitum. After anesthetization, adipose tissue was microdialyzed for 90 min and the perfusates were collected every 30 min. Blood samples were taken at the midpoint of the collection period. Concentrations of glycerol in plasma and perfusates were measured by the fluorometric/colorimetric enzyme method. Total RNA (10 μg) of subcutaneous fat tissues in obese db+/- db+/- (n = 8) and lean db+/- db+/- (n = 8) mice were analyzed by Northern blotting. Representative Northern blots using pooled RNA samples are shown in the insets. Columns and bars represent the mean ± S.E. for the results.

* p < 0.001; ** p < 0.01; *** p < 0.05, lean versus obese, Student’s t test.

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Glycerol concentration in the interstitial fluid of adipose tissue more precisely reflects the amount of glycerol release from adipocytes than that in plasma. Accordingly, we measured the glycerol concentration in the interstitial fluid of adipose tissue by the microdialysis technique. Adipose tissue glycerol concentrations were higher in obese mice than in lean mice (82 ± 11 μM versus 49 ± 5 μM, p < 0.01) (Fig. 3). White adipose AQPap mRNA levels in obese animals were approximately 4-fold higher than those in lean animals (p < 0.001) (Fig. 3). Obese mice also showed elevated HSL mRNA levels (p < 0.05). Thus, AQPap and HSL mRNAs were overexpressed in mice with insulin resistance.

Expression of AQPap mRNA in 3T3-L1 Cells—Next, we examined the time course of glycerol release activity and expression of the genes relating to lipolysis during the differentiation of cultured 3T3-L1 cells. The amount of epinephrine-stimulated glycerol release per hour was measured in 3T3-L1 cells each day after differentiation. Glycerol in the medium became detectable from day 4 after differentiation, increased during differentiation, and reached a plateau level on day 13 (Fig. 4). Undifferentiated 3T3-L1 cells did not express AQPap mRNA. The signal of AQPap mRNA was detectable in the cells on day 3 after differentiation, and increased in parallel with glycerol release activity. It is known that perilipin associates with the surface of intracellular neutral lipid droplets and controls lipolysis (24, 34). The expression patterns of perilipin and HSL mRNAs after differentiation were similar to that of AQPap mRNA (Fig. 4). Neither AQp3 nor AQp9 mRNA were detected in the differentiated 3T3-L1 cells (data not shown).

Hormonal Regulation of AQPap mRNA in 3T3-L1 Cells—AQPap mRNA expression was augmented in the fasted mice with low levels of plasma insulin and suppressed in the refed mice with elevated plasma insulin levels. We hypothesized that AQPap mRNA would be negatively regulated by insulin. The effect of insulin on AQPap mRNA expression was investigated in the differentiated 3T3-L1 cells. Administration of insulin dose-dependently suppressed AQPap mRNA expression in 3T3-L1 adipocytes on day 9 after differentiation (Fig. 5a). Treatment with 10−8 M insulin decreased AQPap mRNA levels by 43%. The time course of AQPap mRNA suppression by insulin was also investigated. Incubation with 10−8 M insulin for 3 h significantly suppressed AQPap mRNA levels and 6 h incubation resulted in 45% suppression (Fig. 5b). Therefore, insulin is a negative regulator of AQPap expression. We also investigated the effect of lipolytic hormones on AQPap mRNA levels. Administration of epinephrine, glucagon, and ACTH, however, did not change AQPap mRNA levels in the differentiated 3T3-L1 cells (data not shown). These results were also consistent with a previously reported finding that these hormones did not affect HSL mRNA levels (35).

Immunological Detection of AQPap Protein in 3T3-L1 Cells and Adipose Tissues—To investigate the regulation and subcellular localization of AQPap at the protein level, we raised an antibody against the carboxyl terminus of AQPap. The specificity of the antibody was assessed by immunohblotting (Fig. 6a). The antibody recognized a single band at 28 kDa corresponding to the predicted molecular mass of AQPap in the crude membrane fraction (M) of differentiated 3T3-L1 adipocytes (lane 4), but not in the soluble cytosolic fraction (C) (lane 3) consistent with the fact that AQPap is an integral membrane protein. The band could not be observed by preimmune serum (lane 2) and was abolished by the presence of excess immunizing synthetic peptide (lane 6). The AQPap protein was detected in the differentiated 3T3-L1 cells (Fig. 6b, lane 2), but not in the undifferentiated 3T3-L1 cells (lane 1). The AQPap protein levels were higher in adipose tissue of the fasted mice (lane 4) compared with those in the fed mice (lane 3). Obese mice showed higher levels of adipose AQPap protein (lane 6) compared with those in lean mouse (lane 5).

Subcellular localization of AQPap protein was studied in the differentiated 3T3-L1 cells by confocal immunocytoscopy. AQPap immunoreactivity was detected in the intracellular region around the nucleus with a scattered pattern (Fig. 6c). When the cells were stimulated by 10−6 M epinephrine for 20 min, the signals in the plasma membrane became robust in comparison with those in the intracellular regions (Fig. 6c), suggesting that AQPap was translocated from intracellular regions to the plasma membrane.

Glycerol Release from 3T3-L1 Cells—The water and glycerol permeability of most aquaporins is inhibited by mercury ions

![Fig. 4. Time course of epinephrine-stimulated glycerol release, AQPap, HSL, and perilipin mRNA expression during differentiation of 3T3-L1 cells.](image-url)
by affecting the cysteine residue near the well conserved NPA (Asn-Pro-Ala) motif, which takes part in the channel forming process (36). We previously reported that the osmotic water permeability in AQPap-expressing Xenopus oocytes was inhibited by HgCl$_2$ and that this inhibition was recovered by the addition of 2-mercaptoethanol similar to the other AQPs (15).

The effect of mercury ions on glycerol release in 3T3-L1 cells was investigated. We tested various concentrations of HgCl$_2$ on epinephrine-stimulated glycerol release from the cells. Administration of HgCl$_2$ at concentrations greater than 10 mM effectively suppressed glycerol release (Fig. 7a). Treatment of 10 mM 2-mercaptoethanol recovered the inhibition of glycerol release between the concentration of 10 and 40 μM of HgCl$_2$ (Fig. 7a). The inhibition of glycerol release by HgCl$_2$ was also confirmed by a separate experiment. Glycerol release was reduced to 19% by the addition of 30 μM HgCl$_2$ (control versus HgCl$_2$, p < 0.0001) and was restored by the supplementation of 2-mercaptoethanol (ME) (HgCl$_2$ + ME versus HgCl$_2$, p < 0.0001) (Fig. 7b). The addition of 2-mercaptoethanol alone did not affect the amount of glycerol released (data not shown). These data sug-
gest that glycerol release from adipocytes is facilitated through AQAP, which is the only detectable aquaglyceroporin in adipocytes.

**DISCUSSION**

Adipose tissue is a major site of glycerol production and secretion (37). Molecules responsible for glycerol release from adipocytes, however, have not yet been elucidated. Recently using the cDNA library from human adipose tissue, we have identified a novel member of the AQP family. As the mRNA of this AQP was expressed most abundantly in adipose tissue, we termed it AQAp (15). Although some members of the AQP family possess permeability to small molecules, including urea and glycerol as well as water, the physiological significance has been obscure. In the current study using mice, we demonstrated that 1) AQAp was the only aquaglyceroporin expressed in adipocytes, 2) fasting enhanced and refeeding suppressed its mRNA expression in adipose tissue, 3) adipose AQAp mRNA was overexpressed in obese mice with elevated adipose glycerol concentration. These data prompted us to hypothesize that AQAp functions as a glycerol channel in adipocytes. This hypothesis is supported by the result that glycerol release from adipocytes was mercury sensitive.

Insulin is the major hormone that suppresses lipolysis (38). Adipose expression of AQAp mRNA was enhanced in fasted mice whose insulin levels in plasma had decreased. Long-term fasting has been found to increase HSL mRNA levels in adipose tissues in rats (39). FATP expression is also augmented by fasting and is thought to facilitate fatty acid efflux from the adipose cell during lipolysis (40). Administration of insulin reduced the expression of AQAP in dose-dependent and time course-dependent manners in 3T3-L1 adipocytes. It had been shown that insulin negatively regulates the expression of HSL and FATP genes (40, 41). Coordinated regulation of HSL, FATP, and AQAp expression will be beneficial for the effective release of FFA and glycerol from adipocytes.

The suppression of AQAP mRNA expression by feeding was impaired in obese mice with insulin resistance. Consistent with this finding, we have observed that the interstitial glycerol concentration in subcutaneous fat tissue was elevated in obese mice using the microdialysis technique. It has been reported that glycerol production in fat tissue was increased in human obesity (33). Hyperglycerolemia is a frequent finding of obesity and is often associated with increased rates of lipolysis (33). Increased glycerol turnover rate in obesity contributes to increased hepatic glucose production, resulting in hyperglycemia (42–44). Impaired suppression of AQAp in the insulin-resistant state may be involved in increased hepatic glucose output through hyperglycerolemia.

In non-stimulated 3T3-L1 cells, AQAP immunoreactivity was detected in the perinuclear cytoplasm with a scattered distribution, suggesting that AQAP resides in the intracellular vesicles. After stimulation of epinephrine for 20 min, the intensity of AQAP signals in the plasma membrane was increased. These findings suggest that AQAP protein was translocated from the intracellular vesicles to plasma membrane. Among the members of the AQP family, AQP2 is located on the principal cells of the renal collecting duct and translocates from the intracellular vesicles to plasma membrane by short exposure to arginine-vasopressin (45). Phosphorylation of AQP2 through the protein kinase A cascade triggers the translocation of AQP2 (46). In adipocytes, binding of epinephrine to adrenergic receptors phosphorylates HSL on several serine residues and activates the enzyme through the protein kinase A pathway (47, 48). Taken together, it is possible that protein kinase A may be involved in the translocation of AQAP.

In conclusion, AQAP is the only aquaglyceroporin that transports glycerol into adipose tissue. Studies on mRNA regulation strongly suggest that AQAP is responsible for the nutritional effect on plasma glycerol and that the disturbed regulation of AQAP mRNA is crucial for hyperglycerolemia in the insulin-resistant state.

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