Enhancement of the Aquaporin Adipose Gene Expression by a Peroxisome Proliferator-activated Receptor γ*κ

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The current study demonstrates that aquaporin adipose (AQPap), an adipose-specific glycerol channel (Kishida, K., Kuriyama, H., Funahashi, T., Shimomura, I., Kihara, S., Ouchi, N., Nishida, M., Nishizawa, H., Matsuda, M., Takahashi, M., Hotta, K., Nakamura, T., Yamashita, S., Tochino, Y., and Matsuzawa, Y. (2000) J. Biol. Chem. 275, 20896–20902), is a target gene of peroxisome proliferator-activated receptor (PPAR) γ. The AQPap mRNA amounts increased following the induction of PPARγ in the differentiation of 3T3-L1 adipocytes. The AQPap mRNA in the adipose tissue increased when mice were treated with pioglitazone (PGZ), a synthetic PPARγ ligand, and decreased in PPARγ−/− heterozygous knockout mice. In 3T3-L1 adipocytes, PGZ augmented the AQPap mRNA expression and its promoter activity. Serial deletion of the promoter revealed the putative peroxisome proliferator-activated receptor response element (PPRE) at −93/−77. In 3T3-L1 preadipocytes, the expression of PPARγ by transfection and PGZ activated the luciferase activity of the promoter containing the PPRE, whereas the PPRE-deleted mutant was not affected. The gel mobility shift assay showed the direct binding of PPARγ-retinoid X receptor α complex to the PPRE, ∆PPARγ, which we generated as the dominant negative PPARγ lacking the activation function-2 domain, suppressed the promoter activity in 3T3-L1 cells, dose-dependently. We conclude that AQPap is a novel adipose-target specific gene of PPARγ through the binding of PPARγ-retinoid X receptor complex to the PPRE region in its promoter.

Glycerol, a product of lipolysis of adipose triglycerides, is an important substrate for hepatic gluconeogenesis (1, 2). Recently, we have demonstrated that aquaporin adipose (AQPap),1 which we cloned as the gene abundantly and exclusively expressed in the human adipose tissue, plays an important role in the secretion of glycerol from the adipose tissue in the normal and the insulin-resistant conditions (3, 4). In the normal mice, the AQPap mRNA was increased by fasting, and decreased by refeeding (3). These changes of AQPap mRNA were efficient to supply the glycerol into the liver for gluconeogenesis in the fasting condition. Recently, we identified negative insulin response element (IRE) in the promoter of human and mouse AQPap (5). The AQPap mRNA amounts were regulated through this IRE by fasting/refeeding. On the other hand, in the insulin resistant mice, the negative IRE of the AQPap gene was not sensitive to the high concentration of plasma insulin at fed stage. This insensitivity resulted in the high expression of AQPap mRNA, the increased concentration of plasma glycerol, and enhanced hepatic glucose production in the insulin resistant mice (3, 5). Therefore, the regulation of AQPap mRNA levels in the adipose tissue is one of the determinant factors for glucose homeostasis in the whole body.

During the course of differentiation of adipocytes, AQPap mRNA was not detectable in the undifferentiated 3T3-L1 preadipocytes, and the mRNA was markedly increased during the differentiation of 3T3-L1 adipocytes (3). The expressions of adipose-specific genes are crucial for the phenotypic determination of the adipocytes (6). The transcriptional factors, which regulate the expression of these adipose-specific genes, include peroxisome proliferator-activated receptor γ (PPARγ 1 and 2) (7, 8), the CCAAT/enhancer-binding protein (6), and sterol-regulatory element binding proteins/adipocyte determination and differentiation factor 1 (6, 9). Above all, PPARγ was shown to be essential for the differentiation of adipocytes (6). PPARγ knockout (−/−) mice had no detectable adipose tissue (10), and the fibroblasts obtained from PPARγ (−/−) embryos failed to differentiate into adipocytes by various stimuli (11, 12). PPARγ forms stable heterodimers with retinoid X receptors (RXR), and this complex binds to a specific DNA sequence, designated peroxisome proliferator-activated receptor response element (PPRE), in the promoter of the target genes (13). PPRE has been identified in the promoters of several genes whose protein products are indispensable for the adipocyte characteristics, such as fatty acid transport protein 1 (FATP1) (14), lipoprotein lipase (15), acyl-CoA synthase (16), and adipocyte lipid-binding protein (17).

FATP1 is one of the fatty acid-transport proteins in the adipocytes (18). Similar to AQPap, the amount of FATP1 mRNA in 3T3-L1 cells was shown to be up-regulated as a consequence of adipose conversion (19). Transcription of

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‡ The abbreviations used are: AQPap, aquaporin adipose; AQP, aquaporin; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor response element; RXR, retinoid X receptor; FATP, fatty acid transport protein; DMEM, Dulbecco’s modified Eagle’s medium; PGC, peroxisome proliferator-activated receptor γ co-activator; IRE, insulin response element; PGZ, pioglitazone; ANOVA, analysis of variance; AF-2, activation function-2; RA, retinoic acid; FCS, fetal calf serum.
FATP1 is activated by PPARγ, through the PPRE in its promoter, in the differentiation process of the adipocytes (14). The free fatty acid released from the adipocytes through the function of FATP1 is another product of lipolysis of the triglycerides and is known to be deeply involved in glucose and lipid metabolism.

In the present study, we demonstrate AQPap as an adipose-specific novel PPARγ target gene, by identifying PPRE in its promoter, showing the transcriptional activation of the gene by PPARγ agonist, and revealing the direct binding of PPARγ-RXRα complex to the AQPap PPRE. The coordinated increases of AQPap and FATP1 gene expressions via the PPREs during adipose-differentiation enable the adipocytes to supply glycerol and free fatty acid for the whole body metabolism.

**EXPERIMENTAL PROCEDURES**

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**Reagents—**Pioglitazone (PGZ) was obtained from Takeda Chemical (Osaka, Japan). Wy14643 was purchased from Calbiochem. 9-cis-Retinoic acid (RA) was obtained from Sigma-Aldrich Japan K. K. (Tokyo, Japan).

**Cells and Animals—**A mouse 3T3-L1 cell line was obtained from Health Science Research Resources Bank (Osaka, Japan). Eight-week-old male C57BL/6J mice (Clea Japan, Inc., Osaka, Japan) were fed powdered chow (CRF-1, Oriental Yeast Inc., Osaka, Japan). The animals were kept at 22 °C with a 12-h dark-light cycle (8 a.m. to 8 p.m.). The animals were acclimatized to the new environment for a week before the experiment. The chow consisted of 53.5% (w/w) carbohydrate, 5.9% (w/w) fat, 23.1% (w/w) protein, and 3.3% (w/w) dietary fibers. At the age of 13 weeks, the mice were divided into two groups. Each group (n = 4, each) was fed either powdered chow or containing 0.01% (w/w) PGZ. Two weeks later, the mice were sacrificed after 12 h of fasting.

Eight-week-old male wild (PPARγ+/+) and heterozygous (PPARγ+/-) PPARγ knockout mice (n = 5 each) (12) were sacrificed after 12 h of fasting and analyzed.

**RNA Analysis—**The total cellular RNA was isolated using the Trizol™ reagent kit (Invitrogen, Tokyo, Japan). The Northern blot analysis was performed as previously described (3, 5). Briefly, 10-μg aliquots of total RNA from the white adipose tissue or 3T3-L1 adipocytes were electrophoresed on 1% agarose/formaldehyde gel and transferred to nitrocellulose membranes. The blots were hybridized with a [32P]dCTP probe using the Multiprime DNA labeling system (Amersham Biosciences, Inc., Buckinghamshire, United Kingdom). After fixation by ultraviolet cross-linking, the filter was prehybridized with QuikHyb hybridization solution (Stratagene) at 65 °C for 0.5 h. Mouse AQPap cDNA (5) and mouse PPARγ cDNA containing the entire coding region were obtained by reverse transcription-polymerase chain reaction using the mice adipose tissue RNA as a template, and then were used as probes for Northern blot analysis. The probes were labeled using the Multiprime DNA labeling system (Amersham Biosciences, Inc.) with [32P]dCTP.

**Gel Electromobility Shift Assays—**To study the binding of the nuclear hormone receptors to the putative PPRE, a double-stranded oligonucleotide, PPREwt, spanning nucleotides –98 to –64 of the mouse AQPap gene upstream sequence was 32P-labeled with polynucleotide kinase (Promega). A 15-μl reaction solution containing end-labeled PPRE oligonucleotide probe (2 × 106 cpm) and 1 μl of in vitro translation reaction was incubated for 20 min at 25 °C and 15 min at 4 °C in a buffer containing 20 mM Hepes (pH 8.0), 60 mM KCl, 1 mM dithiothreitol, 10% glycerol, and 1 μg of poly(dI-dC). The DNA-protein complexes were resolved from the free probe by electrophoresis on a 4% polyacrylamide gel in 0.5× TBE buffer (1× TBE contains 89 mM Tris, 90 mM borate, and 2 mM EDTA). The gels were dried and autoradiographed at ~80 °C. Double-stranded oligonucleotides composed of the following sequences were used for binding and competition analysis. AQPap PPREwt, 5'-TTCTGTGTCTGCTCTCAGGGGAGAGGTCAGTAGG-3'; AQPap PPREmut, 5'-TTCTGTGTCTGCTCTCAGGGGAGAGGtCAGTAGG-3'; PPRE sequence is underlined. The mutated bases are shown in lowercase.

**Plasmids—**The mouse AQPap promoter-luciferase reporter plasmids were constructed by excising the promoter fragment from the genomic clone of AQPap (5) and inserting it into the MluI and XhoI site of the control pGL3 basic luciferase expression vector (Promega). A 0.5-kb DNA fragment of the mouse AQPap gene promoter including the transcription start site (GenBank™ accession no. AB056902) was amplified by PCR using the following primers: forward primer: 5'-TATCATCTTCTCGAGGAGGAGAGGTCAGTAGG-3' and reverse primer: 5'-GCCCTCGAGTTTTCTCAGGGGAGAGGTCAGTAGG-3'. The PCR product was then cloned into the pGL3 vector (Promega, Groningen, Netherlands). The pGL3 vector was a generous gift from Dr. Ronald M. Evans (20). The pGL3 vector was a generous gift from Dr. Ronald M. Evans (20).

**RESULTS**

**Identification of a Putative PPRE in the Promoter of Mouse AQPap Gene—**Fig. 1A shows the expressions of AQPap and PPARγ mRNAs during the differentiation of 3T3-L1 adipocytes. In the undifferentiated state, 3T3-L1 cells had no detectable transcripts of either AQPap or PPARγ. AQPap mRNA expression increased from day 4 after induction of differentiation. PPARγ mRNA induction was seen within 2 days after differentiation of 3T3-L1 cells. Transient transfection assays were performed to knock which promoter region of the mouse AQPap gene is important for induced expression of its mRNA during the differentiation of 3T3-L1 adipocytes (Fig. 1B).

**Statistical Analysis—**The results were expressed as mean ± S.E. The significance of the difference between the groups was evaluated by Student’s t test or analysis of variance (ANOVA) with Fisher’s protected least significant difference test.
Fig. 1. AQPap mRNA expressions and its promoter activities during the differentiation of 3T3-L1 adipocytes. A, AQPap and PPARγ mRNA expressions during the differentiation of 3T3-L1 adipocytes. The total RNAs (10 μg) extracted from 3T3-L1 cells on the indicated day after differentiation-inducing treatment were subjected to the Northern blot analysis for AQPap and PPARγ as described under “Experimental Procedures.” The lower panel represents the ethidium bromide staining of 28 S ribosomal RNAs. B, firefly luciferase constructs containing serial deletions of the mouse AQPap gene promoter (closed bar) or control pGL3 basic (open bar) were co-transfected with pRL-SV40 into 3T3-L1 preadipocytes (left) or differentiated 3T3-L1 adipocytes (right), and assayed for luciferase activities as described under “Experimental Procedures.” The normalized luciferase activities are shown as mean ± S.E. Each value is from three independent experiments. C, upstream sequence of the AQPap gene. The putative PPRE was underlined. D, the putative PPRE sequence in the promoter region of the mouse AQPap gene, compared with the classical PPRE consensus sequence. The bold (uppercase) letters denote conserved base(s).

Inspection of this region of the mouse AQPap gene promoter revealed a putative PPRE of the direct repeat 1 type at −77 to −93, which is similar to consensus PPRE (Fig. 1, C and D) (11–15).

Effects of Thiazolidinedione and PPARγ Deficiency on AQPap mRNA Expression in Mice—Enhancements of AQPap mRNA that occurred subsequent to PPARγ induction during the differentiation of 3T3-L1 cells and the existence of putative PPRE site in its promoter strongly suggested that AQPap is a primary target gene of PPARγ. To elucidate this hypothesis in vivo, we treated mice with 0.01% (w/w) pioglitazone (PGZ), synthetic PPARγ ligand (22) for 2 weeks and measured the mRNA levels of AQPap and PPARγ in the adipose tissue (Fig. 2, A and B). PGZ treatment caused 2.2-fold increase of AQPap mRNA expression in the adipose tissue. As the other side of the spectrum, we examined the AQPap mRNA in PPARγ-deficient mice. The levels of AQPap mRNA expression were significantly decreased in the adipose tissue of PPARγ−/− heterozygous knockout mice (Fig. 2, C and D) (12).

Thiazolidinedione-mediated Induction of AQPap mRNA and Promoter Activity in 3T3-L1 Adipocytes—We investigated the effect of thiazolidinediones on the regulation of AQPap gene transcription in 3T3-L1 adipocytes. Incubation with thiazolidinediones augmented the amount of AQPap mRNA and the promoter activity (−497/+63) (Fig. 3, A and B). To determine the function of the AQPap gene promoter as well as its putative PPRE, transient reporter assays using various AQPap gene promoter constructs were performed in 3T3-L1 adipocytes (Fig. 3C). Serial deletion showed that the basal levels and the PGZ-mediated induction of luciferase activities were highly maintained when the constructs contained the region of −141/−14. The construct −14/+63, which lacked the region of −141/−14, including the putative PPRE, reduced the basal activity and abolished the response to PGZ (Fig. 3C).

To determine the further significance of the putative PPRE in the promoter of AQPap gene, we generated a construct lacking PPRE region (−93/−77) specifically from the wild-type construct (−141/+63) (Fig. 4A), and compared the response to various stimuli between the wild-type (−141/+63) and pAQPap PPRE-deleted constructs (Fig. 4A). Fig. 4B showed the effect of
reporter constructs for 24 h, and luciferase activities were measured (ANOVA). The value of pAQPap-S.E. (set as 1.0. The normalized luciferase activities are shown as mean ± S.E. (n = 3, 3 wells). C, synergistic activation of AQPap gene promoter by PPARγ and RXRα. In 3T3-L1 preadipocytes, 1 μg of pAQPap wild-type (−141/+63) reporter constructs was transfected with or without 500 ng of PPARγ and/or RXRα expression vectors, and treated with or without 10 μM PGZ and/or 9-cis-RA, combination of PGZ and 9-cis-RA, or 10 μM Wy14643 for 24 h before harvest. Luciferase activities were normalized to pRL-SV40 activity, and the value of pGL3-basic vector was arbitrarily set as 1.0. The data are shown as mean ± S.E. (n = 3, 3 wells). C, synergistic activation of AQPap gene promoter by PPARγ and RXRα. In 3T3-L1 preadipocytes, 1 μg of pAQPap wild-type (−141/+63) reporter constructs was transfected with or without 500 ng of PPARγ and/or RXRα expression vectors, and treated with or without 10 μM PGZ and/or 10 μM 9-cis-retinoic acid. After transfection, the cells were incubated for 48 h. The total amount of DNA added was adjusted to 2.01 μg using empty pCDNA3.1. The normalized luciferase activities are shown as mean ± S.E. (n = 3) and are expressed as fold induction relative to the pAQPap wild-type (−141/+63) promoter activity in the pCDNA1 expression vector (empty) (lane 1). The data are shown as mean ± S.E. (n = 3, 3 wells). Similar results were obtained in the other two independent experiments performed in triplicate.

PGZ, 9-cis-RA, and Wy14643, which are the specific ligands for PPARγ, RXRα, and PPARα, respectively, on the luciferase activities of three constructs in 3T3-L1 preadipocytes and adipocytes. These compounds did not induce the reporter activities in the preadipocytes (Fig. 4B, left). PGZ enhanced the luciferase activity of the wild-type construct in 3T3-L1 adipocytes (Fig. 4B, right), similar to the data in Fig. 3B, and further increase was observed when 9-cis-RA was added (Fig. 4B, right). Neither 9-cis-RA nor Wy14643 alone induced the luciferase activity in the wild-type construct (Fig. 4B, right). The pAQPap PPARE-deleted construct had a lower basal luciferase activity, and the responses to the treatment of PGZ were totally abolished (Fig. 4B, right). Fig. 4C clarified the effect of each reagent on the wild-type AQPap gene promoter construct (−141/+63) in 3T3-L1 preadipocytes. The ectopic expression of PPARγ increased the basal luciferase activities of the wild-type construct (lane 9 versus lane 1). PGZ treatment induced the luciferase activities when the preadipocytes were transfected with pAQPap expression construct (lanes 11, 12, 15, and 16). Co-expression of RXR with PPARγ further increased the PGZ-induced luciferase activities (lanes 15 and 16). Addition of 9-cis-RA augmented this increase (lane 12 compared with lane 11, and lane 16 compared with lane 15). These results indicate that the wild-type AQPap promoter activities were enhanced by PGZ in the preadipocyte when they expressed PPARγ.

PPARγ-RXRα Complexes Bind to the AQPap PPARE—PPARγ’s partner for transcriptional activation is RXRα (23). To determine whether PPARγ binds to the AQPap PPARE as complexes with RXRα, gel mobility shift assays were performed with double-stranded oligonucleotides containing the AQPap PPARE (Fig. 5A and C). Transient reporter assays using AQPap PPAREwt or PPAREmut, which is 3-base substitution mutant constructs, were performed in 3T3-L1 preadipocytes (Fig. 5, A and B). These 3-base substitutions have been described previously to diminish the binding of PPARγ to PPARE in the plasmid.
The signal of the $^{32}$P-radiolabeled PPREwt oligonucleotides' binding to PPARγ-RXRα complex in a dose-dependent manner (lanes 5–7). On the other hand, addition of PPREmut oligonucleotides, which were not supposed to bind to the PPARγ-RXRα complex, did not reduce the specific signal (lanes 8–10). These results demonstrate the specific binding of PPARγ-RXRα complex to the AQPap PPRE.

**Generation of Dominant Negative PPARγ Construct and Its Effect on the AQPap PPRE**—To further confirm the PPARγ-dependent enhancement of AQPap gene expression, we generated the dominant negative PPARγ expression constructs (Fig. 6A). In the other nuclear receptor-type transcriptional factors, the mutant construct lacking carboxyl AF-2 domain possessed the dominant negative effect on the transcription of the target genes (24, 25). We generated a mutant PPARγ expression construct, designated ΔPPARγ, which lacked the last 11 amino acids in AF-2 domain (Fig. 6A). As shown in Fig. 6B, the mutant protein derived from ΔPPARγ construct had the ability to bind to the AQPap PPRE oligonucleotides as a complex with RXRα (lane 6), at a similar strength to the wild-type PPARγ protein (lane 4). The specific binding of the mutant PPARγ was abolished when excess amounts of nonlabeled competitor PPREwt oligonucleotides were added (lane 7), similar to the wild-type PPARγ construct (lane 5). Fig. 6C demonstrates that the co-expression of PPARγ and RXRα induced the basal luciferase activity of AQPap promoter in 3T3-L1 preadipocytes (lane 7 versus lane 1), and further increase was observed following the incubation with PGZ (lane 19). These increases of promoter activities were reduced by transfection of the ΔPPARγ construct, in dose-dependent manners (lanes 7–12 and lanes 19–24). In 3T3-L1 adipocytes expressing abundant PPARγ endogenously, the basal AQPap gene promoter activities were inhibited gradually by transfection of the increasing amount of ΔPPARγ construct (Fig. 6D, lanes 1–7). PGZ-induced AQPap gene promoter activity was significantly reduced when these cells were transfected with the increasing amount of ΔPPARγ expression construct (Fig. 6D, lanes 8–14). These data also confirmed the specific activation of AQPap gene transcription by PPARγ.

**The Effect of PPARα-mediated Pathway on the AQPap PPRE**—To elucidate the mechanism by which the promoter activity of AQPap gene was specifically enhanced by PGZ, not by Wy14643 (Fig. 4B), we examined the effect of PPARα-mediated pathway on the AQPap gene promoter. Northern blotting showed that PPARα mRNA was abundantly expressed in liver, but that PPARα mRNA expression was not detectable in mouse white adipose tissue, and 3T3-L1 preadipocytes and adipocytes (Fig. 7A, left). The expression level of AQPap mRNA was not altered in 3T3-L1 adipocytes by the treatment of Wy14643 (Fig. 7A, right). Fig. 7B examined the effect of PPARα expression and Wy14643 on the AQPap promoter activity in 3T3-L1 adipocytes. Without the transfection of PPARα expression plasmid, there was no activation of AQPap gene promoter containing PPRE in adipocytes (Fig. 7B, lane 2 versus lane 1), as was shown in Fig. 4B. When PPARα was exogenously expressed in 3T3-L1 adipocytes, basal luciferase activity of AQPap gene promoter was increased (Fig. 7B, lane 3 versus lane 1). Wy14643 further enhanced the AQPap gene promoter activity (Fig. 7B, lane 4 versus lane 3). These enhanced promoter activities were totally abolished when the PPRE was selectively deleted from the AQPap gene promoter (Fig. 7B, lanes 5–8). Gel mobility shift assay demonstrated the PPARα-RXRα complex had an ability to bind to radiolabeled AQPap PPRE oligonucleotides (Fig. 7C, lane 7 versus lane 6), and this binding was competed specifically by excessive amount of nonradiolabeled AQPap PPRE oligonucleotides (Fig. 7C, lane 8 versus lane 7),
similarly to the specific binding of PPARγ-RXRα complex to AQPap PPRE (lanes 3–5). These data indicate that small amounts of PPARα in adipocytes caused no enhancements of AQPap gene promoter activity through its PPRE by Wy14643, although PPARα had an ability to bind to and activate the AQPap PPRE.

**DISCUSSION**

The current study showed that aquaporin adipose (AQPap) is the adipose-specific novel PPARγ target gene. The amounts of AQPap mRNA and its promoter activity were almost negligible in 3T3-L1 preadipocytes, which did not express PPARγ. Once the cells were differentiated into the adipocytes, PPARγ mRNA and its promoter activity were almost negligible. The AQPap mRNA levels in the adipose tissue were enhanced. The AQPap mRNA levels in the adipose tissue were increased in mice treated with PGZ, a synthetic PPARγ agonist, and decreased in PPARγ−/− heterozygous knockout mice. In 3T3-L1 adipocytes, PGZ augmented the mRNA amount and promoter activity of AQPap gene, although Wy14643, a PPARα agonist, had a trivial effect on the AQPap gene expression. Serial deletion analysis of 5′-flanking promoter region of AQPap gene revealed the putative PPRE site at −485/−73 (Fig. 4). In 3T3-L1 adipocytes, which did not express PPARγ, the promoter activity of AQPap gene was shown in 3T3-L1 preadipocytes and adipocytes, and the increasing amounts of the PPARγ mutant protein in 3T3-L1 adipocytes were transiently transfected with 10 ng of pRL-SV40 plasmids, 500 ng of PPARγ expression vector, the luciferase activity driven by the promoter construct was deprived of the last 11 amino acids in the carboxyl terminus in the AF-2 domain of PPARγ, ΔPPARγ, and ΔPPARγ binds to PPRE-containing oligonucleotides with a similar affinity. The double-stranded probe, PPREwt, was end-labeled with 32P and incubated with in vitro translated RXRα, PPARγ, or ΔPPARγ. The competitor PPREwt was used in 50-fold molar excess. The protein-DNA complexes were analyzed by electromobility shift assay as described under “Experimental Procedures.” C, dose-dependent inhibition of PPARγ-induced AQPap gene promoter activities by ΔPPARγ expression in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were transiently transfected with 10 ng of pRL-SV40 plasmids, 500 ng of pAQPap wild-type lucerase (−141/+63), 200 ng of PPARγ, 500 ng of RXRα, and the increasing amounts of the ΔPPARγ expression vectors for 24 h, and then the medium was supplemented with or without 10 μM PGZ for 24 h before harvest. The total amount of DNA added was adjusted to 2.71 μg using empty pcDNA3.1. The value of pAQPap wild-type lucerase activity in lane 1 was arbitrarily set as 1.0. The normalized lucerase activities are shown as mean ± S.E. (n = 3) in the results, which are represented by a column and bar graph. C, direct and specific binding of PPARα-RXRα complex to the AQPap PPRE. Electrophoretic mobility shift assays were performed as described under “Experimental Procedures.” The pAQPap wild-type lucerase activity in lane 1 was arbitrarily set as 1.0. The normalized lucerase activities are shown as mean ± S.E. (n = 3) in the results, which are represented by a column and bar graph.
PPARγ-induced Transcriptional Activation of AQPap

gene promoter, which indicated the usage of classical PPAR-RXR system for the PPARγ-mediated activation. Recently, we have also identified a PPRE at -62/–46 in the promoter region of the human AQPap gene, which was 100% homologous to that in the mouse AQPap promoter (data not shown). These results indicate that the inducible expression of PPARγ is critical for enhancement of AQPap mRNA in the mouse and human adipose tissue via its PPRE in the promoter.

To date, adipocyte lipid-binding protein (15) is the only gene known to be the direct target of PPARγ and is expressed exclusively in the adipose tissue. In this sense, PPARγ is the second adipose-specific PPARγ target gene. The mRNAs of the other PPAR target genes, including FATP1 (14), lipoprotein lipase (15), acyl-CoA synthase (16), phosphoenolpyruvate carboxykinase (26), liver fatty acid-binding protein (27), acyl-CoA oxidase (28, 29), and hydroxymethylglutaryl-CoA synthase (30), are expressed in the other tissues and organs. There was no sequence specificity in the PPRE of AQPap gene promoter, in comparison with those of other target genes. It remains to be elucidated how the mRNA expression of AQPap is restricted to the adipose tissue. Recently, Dr. Spiegelman and colleagues have isolated two co-activators of PPARγ, designated PPARγ co-activator 1 (31) and 2 (32) (PGC-1 and PGC-2). PGC-1 mRNA is expressed in the brown adipose tissue, heart, kidney, and brain. PGC-1 plays a key role in adaptive thermogenesis, oxidative metabolism, and glucose uptake. PGC-1 can function as a potent transcriptional coactivator for PPARγ. PGC-1 binds to DNA-binding and hinge domains of PPARγ (31). PGC-2, expressed in most tissues, augments the transcriptional and adiogenic activities of PPARγ. PGC-2 binds to AF-1-transactivating domain of PPARγ (32). We also showed that the protein small heterodimer partner, which lacks a DNA-binding domain (33), is also expressed in the adipose cells, and interacts with PPARγ (41). Mutual interaction of all these coactivators for PPARγ may be partially accountable for the adipose-specific expression of AQPap gene through its PPRE.

Mutations within the AF-2 domain, which is located at the carboxyl-terminal ligand binding region, have been shown to abolish ligand-activated transcription in several other receptors including the glucocorticoid receptor (34), estrogen receptor (35), retinoic acid receptor (36), thyroid hormone receptor (37), retinoid X receptor (38), and liver X receptor (39). This domain is likely to be a key region of transcriptional regulation with ligand binding. In this study, we demonstrated that this AF-2 domain is also required for PPARγ activation, for the first time. We confirmed the direct binding of ∆PPARγ-RXRα complex to the PPRE sequence in the AQPap gene promoter, similar to the wild-type PPARγ-RXRα heterodimer (23). Nevertheless, in the mature 3T3-L1 adipocytes, ∆PPARγ repressed the AQPap gene promoter activity by competing with the endogenous PPARγ to form a complex with RXRα and to bind with the PPRE in AQPap gene promoter. These results further confirmed that AQPap is directly regulated by PPARγ-PPRE system.

The differentiated adipocytes actively conduct both synthesis and hydrolyzation of triglyceride. The free fatty acids, the product of triglyceride hydrolyzation, can be re-esterified into triglycerides and re-stored in the adipocytes. However, all glyc-
erols, another product of the triglyceride hydrolyzation, enter the general circulation because of no system to re-uptake into the adipocytes. The released glycerol from the adipocytes becomes a substrate for hepatic gluconeogenesis (1, 2, 40). Therefore, AQPap, the regulator to transport the hydrolyzed glycerol, should be one of the determinant factors for the whole body glucose homeostasis. Our recent study revealed that the transcription of AQPap gene is regulated in response to the nutritional conditions through the negative IRE in its promoter (5). Increased AQPap in the insulin resistant condition was associated with high concentration of glycerol in plasma (3), which might result in the augmentation of hepatic glucose production. Adipose-specific induction of AQPap through PPARγ-PPRE, which is a master regulatory system of the adipocyte phenotype, strongly suggests that the release of glycerol is important as the function of the adipocytes. Coordinated induction of AQPap and FATP1 via PPRE during adipogenesis implies the physiological significance of these pathways supplying glycerol and free fatty acids into the blood. In the differentiation process from preadipocyte to mature adipocyte, insulin might drive the expression of AQPap mRNA probably in an indirect fashion. Further, once the cells are fully differentiated into adipocytes, AQPap gene transcription is negatively regulated directly by insulin through its IRE in the promoter, in accordance with the nutritional conditions. Further analysis on the regulation of AQPap gene transcription should lead to clarification of the mechanism to determine the plasma glycerol concentrations that modify glucose homeostasis.

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PPARγ-induced Transcriptional Activation of AQPap

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