PROGRESSIVE ADIPOCYTE HYPERTROPHY IN AQUAPORIN-7 DEFICIENT MICE: ADIPOCYTE GLYCEROL PERMEABILITY AS A NOVEL REGULATOR OF FAT ACCUMULATION

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Running title: Fat cell hypertrophy in AQP7 null mice
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Aquaporin-7 (AQP7) is a water/glycerol transporting protein expressed in adipocyte plasma membranes. We report here remarkable age-dependent hypertrophy in adipocytes in AQP7 deficient mice. Wildtype and AQP7 null mice had similar growth at 0-16 weeks as assessed by body weight, though by 16 weeks AQP7 null mice had 3.7-fold increased body fat mass. Adipocytes from AQP7 null mice of age 16 weeks were greatly enlarged (diameter 118 µm) compared to wildtype mice (39 µm). Adipocytes from AQP7 null mice also accumulated excess glycerol (251 vs. 86 nmol/mg protein) and triglycerides (3.4 vs. 1.7 µmol/mg protein). In contrast, at age 4 weeks, adipocyte volume and body fat mass were comparable in wildtype and AQP7 null mice. To investigate the mechanism(s) responsible for the progressive adipocyte hypertrophy, glycerol permeability and fat metabolism were studied in adipocytes isolated from the younger mice. Plasma membrane glycerol permeability measured by 14C-glycerol uptake was 3-fold reduced in AQP7 deficient adipocytes. However, adipocyte lipolysis, measured by free fatty acid release and hormone-sensitive lipase activity, and lipogenesis, measured by 14C-glucose incorporation into triglycerides, were not affected by AQP7 deletion. These data suggest that adipocyte hypertrophy in AQP7 deficiency results from defective glycerol exit and consequent accumulation of glycerol and triglycerides. Increasing AQP7 expression/function in adipocytes may reduce adipocyte volume and fat mass in obesity.

INTRODUCTION

The aquaporins are a family of small integral membrane proteins that transport water, and in some cases water and small molecules such as glycerol (‘aquaglyceroporins’) and other small solutes. The aquaglyceroporin AQP3 was cloned initially from kidney and found to transport water and glycerol when expressed heterologously (1-3). AQP3 gene disruption in mice produced a urinary concentrating defect caused by reduced collecting duct water permeability (4), as well as dry skin (5), which was related to impaired epidermal cell glycerol permeability (6). Impaired glycerol transport in AQP3 deficient epidermal cells produced a low glycerol concentration in epidermis and stratum corneum (with normal serum glycerol), resulting in reduced stratum corneum hydration, elasticity and biosynthesis, each of which could be corrected by glycerol replacement therapy (7). These studies provided evidence for a physiological role for aquaporin-facilitated glycerol transport.

The aquaglyceroporin AQP7 was cloned from human adipose tissue (originally named AQPap, ref. 8) and rat testis (9), and shown in heterologous expression systems to function as a water/glycerol transporter. Adipocyte AQP7 expression was found to be sensitive to fasting/refeeding (10), insulin deficiency (11,12),
and steroids and adrenergic agonists (13). These findings provided indirect evidence for a role of AQP7 in adipocyte function. AQP7 is also expressed in testis and sperm (14-16) and kidney (17), where its function is at present unknown.

Here, we investigated a possible role for AQP7-mediated glycerol transport in fat cell physiology by phenotype analysis of transgenic mice with targeted AQP7 gene disruption. During the preparation of our manuscript Maeda et al. (18) reported a mild phenotype of reduced serum glycerol concentration in AQP7 deficient young male mice after adrenergic stimuli and prolonged fasting. Here, we report in older AQP7 null mice marked adipocyte hypertrophy and increased body fat. Mechanistic analysis of adipocyte hypertrophy in AQP7 deficiency suggests pharmacological modulation of adipocyte AQP7 as a possible therapy in obesity.

**METHODS**

**Mice** - AQP7 null mice were generated by targeted deletion of exon 2 (manuscript describing gene knockout procedures in preparation) and backcrossed into a CD1 genetic background. The mice were maintained in air-filtered cages and fed normal mouse chow in the University of California, San Francisco, Animal Care Facility. All procedures were approved by the UCSF Committee on Animal Research.

**RNA and protein analysis** - Total RNA was isolated from epididymal fat and kidney using TRIZOL (Invitrogen). Reverse transcription PCR and quantitative real-time PCR were done using sequence-specific primers (GenBank™ accession numbers: AQP7, NM_007473; peroxisome proliferator-activated receptor-gamma (PPAR-γ), NM_011146; CCAAT/enhancer-binding protein α (C/EBP_), NM_007678). Immunoblotting was done using polyclonal anti-PPARγ and C/EBPα (Santa Cruz Biotechnology, Inc.).

**Fat mass and morphology** - White adipose tissue (WAT) from epididymal or gonadal, mesenteric, and inguinal tissues of wildtype and AQP7 null mice at various ages (4-16 weeks) was excised and weighed. WAT was fixed in formalin, embedded in paraffin, and sections were stained with hematoxylin/eosin. For measurement of diameter, adipocytes were isolated from epididymal fat pad by collagenase digestion (19) and photographing at high magnification for image analysis.

**Glycerol permeability and release** - Glycerol permeability was measured in isolated adipocytes by modification of the method of Ma et al. (5). The adipocyte suspension was incubated for specified times with 100 mM glycerol in Krebs-Ringer Hepes (KRH) containing tracer 3H-glycerol (Amersham Biosciences) at room temperature. After separating on glass fiber filters and washing five times with ice-cold KRH in a suction filtration apparatus, cells were disrupted with 10% sodium dodecyl sulfate. Cell-associated radioactivity was determined by scintillation counting. Protein concentration was measured using a Bio-Rad DC protein assay kit. Glycerol release from WAT was determined as described (20). Epididymal fat tissue was diced and incubated in glucose-free Dulbecco’s modified Eagles’ medium containing 3% fatty-acid free bovine serum albumin (BSA) for 1-3 h. Released glycerol was assayed using a Glycerol Assay kit (Sigma).

**Glycerol, free fatty acids and triglycerides in WAT and serum** - WAT was excised from the gonadal fat pad (female mice, age 16 weeks) and homogenized in PBS. Lipid and aqueous phases were extracted by method of Bligh and Dyer (21). Aliquots of the lipid phase were resolved by thin layer chromatography (TLC) for measurement of free fatty acid (FFA) and triglyceride (TG). Glycerol was assayed enzymatically (Boehringer). For serum analysis, enzymatic assay kits were used for the determination of serum nonesterified fatty acids (Wako), and glycerol, glucose and total TG (Sigma).

**Lipolysis, hormone-sensitive lipase activity and lipogenesis** - Lipolysis was assayed using isolated adipocytes as described (22). Isolated adipocytes were incubated for 1 h at 37 °C in KRH buffer supplemented with 2.5% BSA, and release of FFA was measured. Hormone-sensitive lipase activity was measured using a Lipase kit (RDI) as described (23). Lipogenesis was assayed as described (24). Adipocytes were incubated with [U-14C]-glucose (Amersham Biosciences) in Krebs-Ringer bicarbonate buffer containing 10 nM
insulin and 4% BSA for 1 h at 37 °C. The lipid fraction was extracted, resolved by TLC, and 14C radioactivity in the TG fraction was measured.

**RESULTS**

RT-PCR analysis in Figure 1A confirmed AQP7 transcript expression in adipocytes and kidney from wildtype but not homozygous AQP7 null mice. Analysis of mouse growth through age 16 weeks showed similar mouse body weight, though mouse length was reduced in the AQP7 null mice at 16 weeks (Figure 1B). AQP7 null mice had remarkably greater gonadal fat mass compared to wildtype mice as seen grossly (Figure 1C). Figure 1D (left) shows that fat mass from indicated sites was significantly elevated in both male and female AQP7 null mice at 16 weeks. Epididymal fat mass was comparable in wildtype and AQP7 null mice until age 4 weeks, but became greatly different as the mice aged (Figure 1D, right).

Figure 2A shows histology of gonadal fat at 16 weeks, which was representative of the appearance of fat at multiple sites. Adipocytes were remarkably larger in AQP7 null mice than in wildtype mice. Averaged adipocyte area was increased in AQP7 null mice (5322 ± 157 vs. 2014 ± 67 µm²), suggesting that the greater fat mass in the AQP7 null mice is a consequence of adipocyte hypertrophy.

Figure 2B (top) shows light micrographs of adipocytes isolated from wildtype and AQP7 null mice at age 4 and 16 weeks, which were used for subsequent cell-based studies. While adipocyte size was comparable in mice at age 4 weeks, adipocytes were remarkably enlarged in AQP7 null mice at age 16 weeks. Adipocyte diameter in AQP7 null mice was increased ~3-fold (Figure 2B, bottom).

Figure 2C summarizes the concentrations of glycerol, FFA, and TG in serum and adipocytes from mice at age 16 weeks. Serum parameters were unaffected by AQP7 deletion; similar results were obtained from age 4 week mice (not shown). However, adipocyte glycerol, FFA and TG content, expressed per mg of total protein, were significantly elevated in the AQP7 null mice.

To assess lipolysis, FFA release from isolated adipocytes was measured, as well as the activity of hormone-sensitive lipase, a key regulator of lipolysis. Figure 3C shows that lipolysis was not affected by AQP7 deletion. Lipogenesis was assayed in isolated adipocytes from the incorporation of 14C-glucose into TG. Figure 3D shows similar lipogenesis in wildtype and AQP7 null mice. We also determined whether PPARγ and C/EBPα were induced, which are transcription factors involved in adipogenesis. Immunoblot analysis in Figure 3E shows similar C/EBPα protein expression in wildtype and AQP7 null mice (1.0 ± 0.03 fold protein; 1.1 ± 0.3 fold mRNA, n=5), although PPARγ was slightly reduced in AQP7 null mice at 16 weeks (0.79 ± 0.1 fold protein; 0.42 ± 0.3 fold reduced mRNA, n=5).

**DISCUSSION**

We found marked adipocyte hypertrophy and increased fat mass in AQP7 deficient mice,
which developed progressively after age 6 weeks. Mechanistic studies in isolated adipocytes and minced fat tissue suggested that the ~3-fold reduced glycerol permeability of the adipocyte plasma membrane was responsible for the marked adipocyte hypertrophy in AQP7 deficiency, rather than primary defects in adipocyte lipolysis, lipogenesis, or PPARγ induction (25). We propose that reduced adipocyte glycerol permeability results in intracellular glycerol and TG accumulation, as was verified experimentally, resulting in progressive adipocyte expansion. The recent phenotype study of Maeda et al. (18) did not report data on adipocyte morphology or fat mass, but looked only at relatively young mice focusing on basal and stimulated release of glycerol into the blood. They concluded from adipocyte glycerol release measurements that impaired glycerol ‘gateway’ function was responsible for the phenotype of reduced plasma glycerol. However, although we agree that adipocyte glycerol permeability is impaired in AQP7 deficient adipocytes (from glycerol uptake measurements in Figure 3A), glycerol ‘gateway’ function cannot be deduced from the glycerol release measurements of Maeda et al., which depend on glycerol permeability as well lipolysis rate and steady-state adipocyte glycerol content. Increased adipocyte size and fat mass in AQP7 deficient mice were not noted by Maeda et al., possibly because the phenotype of older mice was not studied.

Figure 3F shows a proposed mechanism for progressive TG accumulation in AQP7 deficient adipocytes. Reduced plasma membrane glycerol permeability in AQP7 deficiency results in an increase in steady-state glycerol concentration in adipocyte cytoplasm. The 3.0-fold reduced plasma membrane glycerol permeability is consistent with the 2.9-fold increase in steady-state adipocyte glycerol concentration. Increased adipocyte glycerol concentration would then increase glycerol 3-phosphate and hence TG biosynthesis.

Our results thus focus attention on adipocyte glycerol permeability as a novel regulator of adipocyte size and whole body fat mass. Modulation of adipocyte AQP7 expression and/or function may thus alter fat mass, providing a rational basis for investigation of AQP7 upregulation as therapy in some forms of obesity.

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REFERENCES


**FIGURE LEGENDS**

**Figure 1. Increased fat tissue content in AQP7 deficient mice.** A. RT-PCR of white adipose tissue (WAT) and kidney of wildtype (+/+) and AQP7 null (-/-) mice, amplifying full-length AQP7 transcript. B. Mouse weight and length (excluding tail) at indicated ages (SE, 5-7 mice). *, p < 0.05. C. Photographs of mice showing increased gonadal fat in AQP7 null mice at age 16 weeks (arrows). D. (left) Total and region-specific fat mass in male and female mice (SE, 6 mice) at age 16 weeks. (right) Age-dependent epididymal fat mass (SE, 6 mice). *, p < 0.05. ***, p < 0.01

**Figure 2. Adipocyte hypertrophy in AQP7 deficiency.** A. Histology of gonadal fat (stained with hematoxylin and eosin). Bar, 100 µm. B. (top) Micrograph of adipocytes isolated from epididymal fat of mice of indicated age and genotype. Bar, 100 µm. (bottom) Averaged adipocyte diameter (SE, 150 cells analyzed). *, p < 0.01. C. Glycerol, free fatty acid (FFA), and triglyceride (TG) content in serum (top) and adipocytes (bottom) from mice of age 16 weeks (SE, 6-8 mice). *, p < 0.05. ***, p < 0.01.

**Figure 3. Adipocyte glycerol transport and fat metabolism.** A. 14C-glycerol uptake at 5 min in suspensions of adipocytes from mice at age 6 weeks (individual mice shown along with mean ± SE). *, p < 0.01. Inset shows linearity of uptake. B. Glycerol release from minced adipose tissue (SE, n=6). *, p < 0.01. C. Lipolysis. (top) Released FFA from adipocytes. (bottom) Hormone-sensitive lipase activity (SE, n=6). D. Lipogenesis. Incorporation of 14C-glucose into TG fraction (SE, n=5). E. Immunoblot
analysis of PPARγ and C/EBPα. Data are representative of five separate samples. F. Proposed mechanism for adipocyte hypertrophy in AQP7 deficiency.
Figure 1
Figure 2

A

B

C

serum

glycerol

FFA

TG

adipocyte

glycerol

FFA

TG

adipocyte diameter (µm)

adipocyte diameter (µm)
Figure 3