

AQP7 Up-Regulation in the Skeletal Muscles of Mice with Diet Induced Obesity

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Abstract

Aquaporin (AQP) 7 and AQP9 are membrane proteins and are the members of aquaglyceroporin which transports glycerol in addition to water molecule. Glycerol is a direct source of glycerol-3 phosphate for the synthesis of triglycerides. We thought that the expression of AQP7 and AQP9 would be altered in the skeletal myofibers in mice with diet-induced obesity (DIO) as compared with that of control chaw-fed mice. RNA and protein levels of AQP7 and AQP9 were studied in the quadriceps femoris muscles of mice with DIO and normal control mice. Real time quantitative RT-PCR analysis showed that mouse AQP7 mRNA levels in skeletal muscles were significantly higher in mice with DIO than in normal control mice ($P < 0.01$); whereas mouse AQP9 mRNA levels were not different between the two groups ($P > 0.05$). Histochemically the myofibers of mice with DIO contained numerous lipid droplets in oil red O stain samples. Immunohistochemical study of DIO mouse muscles showed enhanced expression of AQP7 at the myofiber surface membranes; while AQP9 expression appeared to be similar to that of normal control mice. These findings imply that the up-regulated expression of AQP7 in DIO mouse muscles facilitates the secretion of glycerol from myocytes.

Keywords: AQP7; AQP9; RNA and protein levels; Skeletal muscles; Diet induced obese mouse

Introduction

Obesity is one of the clinical manifestations of metabolic syndrome which elicits type 2 diabetes, non-alcoholic fatty liver disease and cardiovascular disorders [1-3]. Generally speaking in human obese individuals, lipids accumulate not only in adipose tissue but also in non-adipose tissue such as liver and skeletal muscle. In fact it is reported that diet induced obesity significantly increased the triacylglyceride content in muscle compared with chow-fed control animals [4]. Lipids accumulated in the skeletal muscle cells are mainly composed of triglyceride which are synthesized by glycerol and long chain fatty acids via glycerol-3 phosphate.

Aquaporins (AQPs) are the small intrinsic channel-forming membrane proteins of epithelial and endothelial cells and are divided into two groups [5]. One group of AQPs is water selective transport channel and the other group of AQPs is a water channel permeable to neutrally charged small molecules such as glycerol, urea, and purines [6,7]. In addition, AQP7 and AQP9 transport arsenite as well [8]. The latter AQPs are called aquaglyceroporins in which AQP3, AQP7, AQP9 and AQP10 are included [5,9]. Glycerol is a direct source of glycerol-3 phosphate for the synthesis of triglyceride [10] which is the main component of neutral lipid. AQP7 and aquaporin adipose (AQPap) were independently cloned from rat testis [6] and mouse fat tissue [11], respectively. AQP7 was a rat homologue for AQPap [6,11]. AQP7 is thought to function as the gateway for the efflux of lipolysis derived glycerol from adipocytes, while AQP9 is thought to play a role in the influx of circulating glycerol into the hepatocytes [12,13].

Previously we examined the aquaglyceroporins (such as AQP7 and AQP9) expression in the skeletal muscles of genetically obese leptin-deficient *ob/ob* mice [14] and we found the up-regulated expression of AQP7 in the skeletal muscles of these mice [14]. The plausible mechanism of AQP7 up-regulation in the skeletal muscles of these mice may be due to the leptin deficiency, since the leptin down-regulates

AQP7 expression through the phosphatidylinositol 3-kinase /Akt / mammalian target of rapamycin pathway [15]. Currently obese humans are increasing and human obesity is a risk factor of lifestyle-related diseases such as cardiovascular disease, stroke and fatty liver. Therefore it is important to study the pathophysiology of diet induced obesity. In this study we were interested in the expression of AQP7 and AQP9 in the skeletal muscles of non-genetical, diet induced obese mice and performed the expression analyses of these AQPs at RNA and protein levels.

Materials and Methods

Experimental animals

Male C57BL/6J mice were obtained from Sankyo Labo Service Corporation (Tokyo, Japan) at 5 weeks of age and fed a normal laboratory diet for 1 week to acclimatize the animals to their new conditions. Then the mice were divided into 2 groups ($n=7$ in each group): the control chow-fed group mice were fed by normal laboratory diet; while the mice with DIO received a high-fat diet (Research Diets D12451, 45 energy % of fat) ad libitum for 16 weeks to induce obesity. Animals were reared in a room with controlled temperature ($20 \pm 2^\circ\text{C}$), humidity ($55 \pm 15\%$), and a 12-h day cycle (8:00 AM – 8:00 PM). The feed was changed 15:00 every day. Seven mice with DIO (C57BL/6J) and seven control chow-fed mice were sacrificed by cervical dislocation and the quadriceps femoris muscles were excised from each mouse in

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order to analyze the mRNA levels of AQP7, AQP9 and 18s ribosomal RNA. Among these muscles, 5 quadriceps femoris muscles from mice with DIO and control chow-fed mice, respectively, were served as histological and immunohistochemical study. All animal studies were conducted in accordance with the "Standards Relating to the Care and Management of Experimental Animals" (Notice No. 6 of the Office of Prime Minister dated March 27, 1980) and with approval from the Animal Use Committee of Showa University.

Quantitative real time reverse transcription polymerase chain reaction (real-time RT-PCR) for AQP7 and AQP9 mRNAs in skeletal muscles of diet induced obese mice and chow-fed mice

By using TRIzol (Invitrogen), total RNA was extracted from each muscle of 7 obese mice with DIO and 7 age matched control chow-fed mice. Mouse AQP7 and mouse AQP9 mRNA concentrations were estimated by real-time RT-PCR.

By using Affinity Script QPCR cDNA Synthesis Kit (Agilent Technologies) and 100 ng of extracted total RNA, first-strand cDNA was synthesized. Based on the mouse AQP7 [Mus musculus AQP7, MA101958, TaKaRa], mouse AQP9 [Mus musculus AQP9, MA117402, TaKaRa] sequences: mouse AQP7 and AQP9 mRNA oligonucleotide primer sets were designed. The mouse AQP7 mRNA: sense strand, 5'-TGGGTTTTGGATTCCGAGT-3'; antisense strand, 5'-TGTTCTTCTGTGCGGTGATGG-3'; and the mouse AQP9 mRNA: sense strand, 5'-CTCAACTC TGGTTGTGCCATGAA-3'; antisense strand, 5'-ATCATA GGGCCCACGACAGGTA-3'. To compensate for differences in RNA quality or RT efficacy, mRNA expression of mouse 18s ribosomal RNA was calculated in each muscle. Oligonucleotide primers for 18s ribosomal RNA were designed: sense strand, 5'-GATCCGAG GGCCTCACTAAAC-3'; antisense strand, 5'-AGTCCCTG CCCTTTGTACACA-3'. For the measurement of mouse AQP7, 9 and mouse 18s ribosomal RNA levels, each primer set was mixed with the respective cDNA using SYBR Prime Script RT-PCR Kit II (TaKaRa). By using an ABI Prism 7900 sequence detection system (Applied Biosystems), real-time PCR was performed with thermocycle conditions including initial denaturation at 95°C for 30 sec followed by 45 cycles at 95°C for 5 sec, 60°C for 1 min with dissociative reaction of PCR products from 65°C to 95°C. By determining the cycle number, threshold cycle was calculated utilizing the Sequence Detector Systems version (Applied Biosystems). Mouse AQP7 and AQP9 mRNA expressions were measured using the standard curve method normalizing with that of mouse 18s ribosomal RNA internal control. Total RNAs from muscles of 7 mice with DIO and 7 chow-fed control mice were analyzed in duplicate by this method and the mean was calculated in each muscle.

Peptide synthesis and antibody production

The previously described general procedures were used for peptide syntheses and antibody production [16,17]. Briefly, the peptide (MVQASGHRRSTRGSK-C) of the N-terminal end of the cytoplasmic domain in the human AQP7 molecule (Entrez NM001170) and the peptide (KAEQSEDKPEKYE-C) of the C-terminal end of the cytoplasmic domain in the human AQP9 molecule (Entrez NM020980) were respectively synthesized and the extra cysteine was added to the C-terminus of AQP7 and AQP9 molecules. At an extra cysteine residue in each peptide, bovine thyroglobulin was added. The antibodies against these peptides were generated in rabbits. By solid phase enzyme-linked immunosorbent assay, the rabbit polyclonal antibody titers were measured, which were $\times 102,400$ for both AQP7 and AQP9. These

antisera were affinity purified. The affinity purified rabbit polyclonal AQP4 antibody against the C-terminal end of the rat AQP4 was also previously produced in our laboratory [18].

Histopathological and immunohistochemical studies

The quadriceps femoris muscles were excised from 5 mice with DIO and 5 control chow-fed mice after cervical dislocation, and were immediately frozen in isopentane cooled with liquid nitrogen. Frozen cryostat muscle cross sections with 6- μ m thickness were placed on slide glasses and stained with hematoxylin and eosin (HE) and oil red O solutions, respectively. The immunohistochemical stainings with anti-AQP7 and AQP9 antibodies, respectively, were also performed as follows. Non-specific reaction was eliminated by incubating the sectioned muscle samples with 5% normal swine serum in phosphate buffered saline (PBS) for 20 min at room temperature. Then the affinity-purified primary rabbit anti-AQP7 and AQP9 antibodies were diluted to a final IgG concentration of 5 μ g/ml, and were applied, respectively, to the sectioned muscle samples during overnight at 4°C. By applying normal rabbit IgG at a concentration of 5 μ g/ml instead of the respective primary antibody, the negative control specimens were made. After washing, the sections were incubated with FITC-conjugated swine anti-rabbit immunoglobulin (DAKO code No. F0205, Denmark) diluted 1:50 in PBS for 60 min at room temperature. For the judgment of myofiber type [19], serial sections of HE or oil red O stained muscle samples and the immunostained muscle samples with anti-AQP7 or AQP9 antibody from obese DIO or control chow-fed mice were also immunostained with the affinity purified rabbit anti-AQP4 antibody at the IgG concentration of 5 μ g/ml [18]. The immunostained muscles were examined using Nikon H 550L fluorescent microscope.

Statistical analysis

The data presentation was done in group mean \pm standard error of the mean. The difference between DIO mouse group and wild chow-fed mouse group was evaluated by two-tailed t test. A p-value less than 0.05 was considered to be statistically significant.

Results

Quantitative real time RT-PCR for AQP7 and AQP9 mRNAs in the skeletal muscles of obese mice with DIO and control chow-fed mice

The standard curves for the quantification of mouse AQP7 and AQP9 mRNAs were linear across 4 to 5 log ranges of RNA concentration. Correlation coefficients were 0.9642 for mouse AQP7 mRNA, 0.9805 for mouse AQP9 mRNA and 0.9952 for mouse 18s ribosomal RNA. Group mean ratios \pm standard error of the mean of mouse AQP7 mRNA copy number versus mouse 18s ribosomal RNA copy number were 190.6 ± 36.9 and 100.0 ± 21.1 in the skeletal muscles of 7 mice with DIO and 7 control chow-fed mice, respectively. These two ratios were statistically significantly different ($P < 0.01$ two-tailed t-test) (Table 1). On the other hand, group mean ratios \pm standard error of the mean of mouse AQP9 copy number versus mouse 18s ribosomal RNA mRNA copy number in the skeletal muscles of 7 mice with DIO and 7 control chow-fed mice were 86.7 ± 15.9 and 100.0 ± 21.6 , respectively. These ratios were statistically non-significant ($P > 0.05$ two tailed t test) (Table 1).

Histopathological and immunohistochemical studies

The cross-sectioned muscle tissues with HE staining of control chow-fed mice contained polygonal shape myofibers without variation of myofiber size. Cytoplasmic appearances had two types

	mouse AQP7 or AQP9 mRNA copy number		mouse 18s ribosomal RNA copy number	
	AQP7	AQP9	AQP7	AQP9
Mice with DIO (n=7)	190.6 ± 36.9*	86.7 ± 15.9*	P<0.01	P>0.05
Control chow-fed mice (n=7)	100.0 ± 21.3	100.0 ± 21.6		

* Group mean ± standard error of the mean; P values were calculated by two-tailed t-test.

Table 1: Quantitative real time RT-PCR for AQP7 and AQP9 mRNAs in the quadriceps femoris muscles of mice with diet induced obese (DIO) and control chow-fed mice.

of characteristics: a smooth cytoplasmic appearance and a somewhat granular appearance with slit-like structures (Figure 1A). HE stained DIO muscles showed the similar findings to those of control muscles and somewhat different features from the muscles of control chow-fed mice. Muscle tissues of mice with DIO revealed the variation of myofiber size and contained scattered small angulated myofibers and apparently degenerating myofibers in which non-homogeneous sarcoplasmic appearance, slit like structures and /or surface indentation were observed (Figure 1B).

Muscle tissues with oil red O staining of mice with DIO contained scattered myofibers with numerous lipid droplets (Figure 2A). Immunostaining of serial muscle section with anti-AQP4 antibody showed that most of these lipids containing myofibers were immunonegative, and therefore slow twitch type 1 fibers (Figure 2B). The myofibers with numerous lipid droplets were frequently observed in the mice with DIO as compared with control chow-fed mice (Figure 2C).

Immunohistochemical staining of skeletal muscles of DIO mice with primary anti-AQP7 antibody demonstrated that most of the myofibers were positively stained at their myofiber surfaces with slight variation of staining intensity (Figure 3A). Intensely AQP7 stained myofibers were type 2 fibers as judged by the serial muscle section immunostained with primary anti-AQP4 antibody (Figure 3B). Immunohistochemistry of skeletal muscles of control chow-fed mice with primary anti-AQP7 antibody contained both immunopositive and immunonegative myofibers at their cell surfaces (Figure 3C). Based on these observations, immunohistochemistry of the skeletal muscle with anti-AQP7 antibody showed that immunostaining intensity is stronger in mice with DIO than that of control chow-fed mice. Immunostaining muscle specimens with normal rabbit IgG instead of the primary antibody revealed no immunostaining (Figure 3D).

Immunohistochemical staining of skeletal muscles of DIO mice with primary anti-AQP9 antibody showed the scattered immunopositive myofibers at their cell surfaces (Figure 4A). Serial muscle section immunostained with anti-AQP4 antibody revealed that the immunopositive myofibers with anti-AQP9 antibody were also immunopositive with anti-AQP4 antibody (Figure 4B). These myofibers were, therefore, type 2 myofibers. Immunohistochemistry of skeletal muscles of control chow-fed mice with anti-AQP9 antibody showed the similar findings to those of DIO mouse muscles stained with anti-AQP9 antibody (Figure 4C).

Discussion

We recently reported that the up-regulated expression of AQP7 at RNA and protein levels in the skeletal muscles of leptin deficient obese *ob/ob* mice [14]. Leptin is a 16kDa peptide hormone secreted by adipocytes and regulates several physiological processes such as

glucose and lipid metabolism, blood pressure homeostasis, immunity and reproduction [12,20].

Recently leptin has been shown to down-regulate the AQP7 function through the phosphatidylinositol 3 kinase/Akt/mammalian target of rapamycin pathway in human visceral adipocytes and hepatocytes [15]. We thought that up-regulated expression of AQP7 in the skeletal muscles of obese leptin deficient *ob/ob* mice is mainly depending on their lack of leptin. However in obese humans, Considine et al. reported that serum immunoreactive leptin levels were up to four times higher than in normal-weight subjects [21]. In animal experiments, even in isocaloric diets, leptin levels were higher in rat group fed with either the high fat diet or the high protein diet compared with those fed with the high carbohydrate diet [22]. In addition leptin and insulin concentrations of mice with DIO were also described to be high in comparison with those of control mice [23,24]. Even under the circumstances of the elevated concentrations of leptin and insulin, both of which suppress the expression of AQP7 [15,25,26] through the



Figure 1: Hematoxylin and eosin (HE) staining of the cross-sectioned quadriceps femoris muscles of control chow-fed mouse (A) and mouse with diet induced obesity (DIO) (B). In control mouse muscle (A), the scattered myofibers (arrow) with slight granular sarcoplasmic appearance and slit like structures are seen. The DIO muscle (B) contains scattered angulated small diameter myofibers (arrow head) and apparently degenerating myofibers (arrow) in which non-homogeneous sarcoplasmic appearance, slit like structures, and / or surface indentation are observed. Bar=50 μm (A,B).

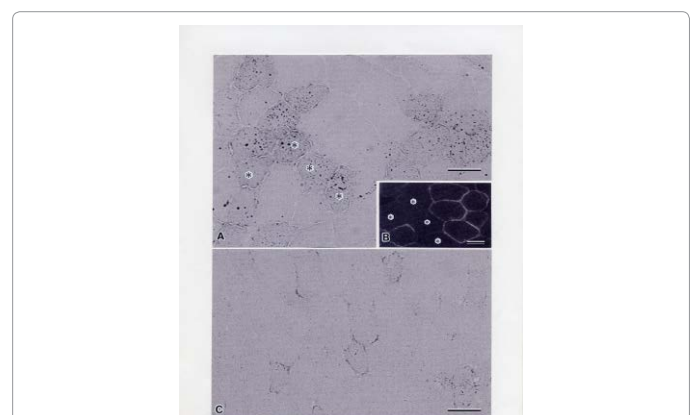


Figure 2: Oil red O staining muscle of mouse with DIO (A) and serially sectioned muscle immunostained with anti-AQP4 antibody (B). The DIO muscle (A) contains a lot of myofibers with numerous lipid droplets in their sarcoplasm. These myofibers (asterisk) in A are type 1 fibers, since same myofibers (asterisk) in serial muscle section (B) are immunonegative with anti-AQP4 staining. The lipid droplets in the DIO myofibers are more numerous than those of oil red O stained control muscle (C). Bar=50 μm (A, B,C).

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