AQP4-Dependent Water Transport Plays a Functional Role in Exercise-Induced Skeletal Muscle Adaptations

Davide Basco1, Bert Blaauw2,3, Francesco Pisani1, Angelo Sparaneo1, Grazia Paola Nicchia1, Maria Grazia Mola1, Carlo Reggiani3,4, Maria Svelto1, Antonio Frigeri1,*

1Department of Bioscience, Biotechnologies and Biopharmaceutics and Center of Excellence in Comparative Genomics (CEGBA), University of Bari, Bari, Italy, 2Venetian Institute of Molecular Medicine, Padua, Italy, 3Department of Biomedical Sciences, University of Padua, Padua, Italy, 4CNR, Institute of Neuroscience, Padua, Italy

Abstract

In this study we assess the functional role of Aquaporin-4 (AQP4) in the skeletal muscle by analyzing whether physical activity modulates AQP4 expression and whether the absence of AQP4 has an effect on osmotic behavior, muscle contractile properties, and physical activity. To this purpose, rats and mice were trained on the treadmill for 10 (D10) and 30 (D30) days and tested with exercise to exhaustion, and muscles were used for immunoblotting, RT-PCR, and fiber-type distribution analysis. Taking advantage of the AQP4 KO murine model, functional analysis of AQP4 was performed on dissected muscle fibers and sarcolemma vesicles. Moreover, WT and AQP4 KO mice were subjected to both voluntary and forced activity. Rat fast-twitch muscles showed a twofold increase in AQP4 protein in D10 and D30 rats compared to sedentary rats. Such increase positively correlated with the animal performance, since highest level of AQP4 protein was found in high runner rats. Interestingly, no shift in muscle fiber composition nor an increase in AQP4-positive fibers was found. Furthermore, no changes in AQP4 mRNA after exercise were detected, suggesting that post-translational events are likely to be responsible for AQP4 modulation. Experiments performed on AQP4 KO mice revealed a strong impairment in osmotic responses as well as in forced and voluntary activities compared to WT mice, even though force development amplitude and contractile properties were unvaried. Our findings definitively demonstrate the physiological role of AQP4 in supporting muscle contractile activity and metabolic changes that occur in fast-twitch skeletal muscle during prolonged exercise.

Citation: Basco D, Blaauw B, Pisani F, Sparaneo A, Nicchia G, et al. (2013) AQP4-Dependent Water Transport Plays a Functional Role in Exercise-Induced Skeletal Muscle Adaptations. PLoS ONE 8(3): e58712. doi:10.1371/journal.pone.0058712

Editor: Jose A. L. Calbet, University of Las Palmas de Gran Canaria, Spain

Received October 18, 2012; Accepted February 5, 2013; Published March 8, 2013

Copyright: © 2013 Basco et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Funding was provided by the Association Française Contre les Myopathies (project no. 14336 to AF) and FIRB - Rete Nazionale di Proteomica (RBRN07BMCT_009). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: a.frigeri@biologia.uniba.it

Introduction

Regulation of cell volume is an essential property of all animal cells. In skeletal muscle, exercise is associated with a wide range of cellular changes that would be expected to influence cell volume. These complex electrical, metabolic and osmotic changes strongly affect individual factors regulating muscle volume despite their likely importance during exercise. One of the major aspects of cell volume control is represented by metabolically dependent processes that directly balance the passive solute and water fluxes, which would otherwise be expected to cause cell swelling under conditions of intracellular membrane-impermeant solutes [1].

In skeletal muscles, fast-twitch myofibers express the mercurial insensitive water channel aquaporin-4 (AQP4)[2]. AQP4 is expressed as two major isoforms of 32 kDa (AQP4-M1) and 30 kDa (AQP4-M23), which differ by 22 amino acids in the N-terminus [3]. These two major AQP4 isoforms are organized in the plasma membrane in higher order structures called Orthogonal Array of Particles (OAPs) [4,5,6] whose expression is affected in several muscular dystrophies [7,8,9,10,11]. The dimension of an OAP is tightly associated to the M1/M23 AQP4 isoform ratio, given that M23 is the OAPs-forming isoform, and M1 alone is unable to form OAPs [5].

We previously postulated that AQP4, together with the endothelial AQP1, may promote water exchange between blood and muscle fibers in order to sustain the volume changes occurring during muscle activity, which may be related to the substantial muscle swelling and intracellular osmolyte production occurring during exercise [12,13,14]. Consistently with this hypothesis, our recent work based on differential 2D Blue Native/SDS-PAGE on quadriceps muscles from WT and AQP4 KO mice demonstrated that the ablation of AQP4 alters metabolic pathways directly involved in energy metabolism and calcium handling [15].

Although the physiological relevance of this water channel in the skeletal muscle is still not well defined or even relegated to “a vestigial remnant from an ancient time” [16], some evidence suggests a potential physiological role of AQP4 in skeletal muscle since muscle activity modulates AQP4 expression [17,18]. To shed light on the relevance of AQP4 for skeletal muscle function, in this study we examined the effect of endurance training on AQP4 protein levels in skeletal muscles of rats. Furthermore, we analyzed whether the ablation of AQP4 affects fiber osmotic behavior and physical activity (forced and voluntary) in AQP4 KO mice. Finally, we measured contraction parameters in vivo and ex-vivo on fast-twitch muscles of WT and AQP4 KO mice.
The data presented here indicate a pivotal physiological role of AQ44 in skeletal muscle, in both basal and training induced muscle activity.

**Results**

Endurance exercise increases AQ44 expression in rat fast-twitch skeletal muscle

To determine whether endurance exercise has an effect on AQ44 protein expression in skeletal muscle, we performed immunoblot analysis on different rat skeletal muscle lysates after 10 (D10) and 30 (D30) days of treadmill exercise compared to age and sex-matched sedentary (sed) rats. We selected 4 fast-twitch muscles, tibialis anterior (TA), extensor digitorum longus (EDL), quadriceps (QUAD) and flexor digitorum brevis (FDB) and the slow-twitch soleus (SOL) muscle. Among the muscles of the first group, FDB muscle represented an exception to the strong relationship between AQ44 expression and fast-twitch fibers [19].

Fig. IA reports the performances obtained by rats subjected to endurance exercise compared with the first bout of activity (D1). Rats at D1 ran about 400 m; at D10, the daily mean distance significantly rose to 1230 m and at D30, rats considerably improved daily performances, covering a mean distance of about 3000 m. The effects of exercise on AQ44 protein levels are reported in fig. IB-C: ten days of endurance exercise produced a strong increase in AQ44 protein levels in TA (+91.4 ±12.4%), EDL (+80 ±6.3%) and QUAD (+82.1 ±7.3%) muscles, and at D30 the expression did not significantly change compared to D10. Interestingly, in FDB muscle we did not find any significant variation in AQ44 expression after 10 or 30 days of exercise. Predictably, the expression of AQ44 in SOL muscle was not detectable before or after the exercise. In all the immunoblotting experiments, protein levels were corrected for whole protein variation in AQ44 expression after 10 or 30 days of exercise.

A QUAD mice subjected a group of 8 rats to 5 days (D5) of treadmill activity. Western blot analysis performed on QUAD muscles revealed that five days of exercise did determine, although not statistical significant, an increase in AQ44 protein levels compared to sed rats (Fig. 1E-F). However, statistical analysis confirms that AQ44 over-expression become significant after ten days of exhaustive exercise.

We further investigated the possible role of stress linked to exercise on muscle AQ44 expression [23]. To this purpose, animals (classified as “stressed-sed” – SS) were placed on the treadmill for 15 min each day for 5 (SS5; n = 8) and ten days (SS10; n = 6) without performing any forced activity. We then compared QUAD muscle lysates of sed, SS5, SS10 and D10 rats by Western blot analysis. Results reported in Fig. 2A-B showed that AQ44 protein levels in SS5 and SS10 groups remained the same as those observed in sed group, excluding any involvement of stress in muscle AQ44 increase during exercise.

Finally, to assess whether the increase in AQ44 protein expression after treadmill exercise is determined by an increase in the relative mRNA expression, we examined AQ44 transcript copy numbers in QUAD muscles from sed and D10 rats. Quantitative Real Time-PCR demonstrated that levels of AQ44 mRNA did not change after treadmill exercise (Fig. 2G), suggesting that post-transcriptional events are likely to be responsible for the increase in the expression of the protein. AQ44 protein over-expression after endurance exercise is fiber type independent

To investigate whether the increase in AQ44 protein after 10 and 30 days of endurance exercise was a consequence of changes in skeletal muscle fiber type distribution, MHCs immunostaining analysis was performed in QUAD, SOL and FDB muscles, and the percentage of AQ44-positive fibers in the same muscles was detected. As shown in Fig. 3, no significant changes in the percentage of MHCs-fiber type distribution were observed in any of the examined muscles. Interestingly, the percentage of AQ44-positive fibers did not change after exercise, even if a significant increase in AQ44 protein levels at both D10 and D30 was found (see fig. 1). Such an increase was not detectable in the immunostained cryosections, likely because of the lower sensitivity of the immunofluorescence compared to Western blot [24].

These results clearly show that the increase in AQ44 protein levels induced by endurance exercise is independent of fiber type transition and is not due to an increased number of fibers expressing AQ44.

AQ44 is over-expressed in WT mice quadriceps following endurance exercise

To test whether exercise had the same effects on mice skeletal muscle, we performed AQ44 immunoblot analysis of quadriceps muscles from AQ44 WT mice after 10 and 30 days of both forced and voluntary activity, compared with age and sex-matched sed mice. Fig. 4 shows a significant increase in AQ44 expression after 10 days of treadmill exercise compared to sed mice (+74.6 ±8.3%; p<0.05 vs sed), which rose to +129.8 ±18.9% at D30 (p<0.05 vs sed). The same effect on AQ44 modulation was observed after voluntary activity, with an increase in AQ44 protein levels comparable to those observed in treadmill mice at D10 (+58.4 ±6.1%; p<0.05) and at D30 (+75.8 ±5.4%; p<0.05) compared to sed mice.

These results suggest that physical exercise positively modulates AQ44 expression in mouse fast-twitch skeletal muscle in both the paradigms. Furthermore, these data were consistent with those obtained in rats and confirm the involvement of AQ44 water channels in skeletal muscle adaptation to activity.
Figure 1. AQP4 protein expression after endurance exercise in rats. A) Daily mean distance at day 1 (D1), day 10 (D10) and day 30 (D30). Note that rats significantly increased performance during the training period; n = 6-8 per group. ***p < 0.0001 vs D1. **p < 0.001 vs D1. *p < 0.01 vs D1. B) Immunoblotting analysis of AQP4 protein expression on TA, EDL, QUAD, FDB and SOL muscles. Note the presence of the two AQP4 isoforms of 30 and 32 kDa. Except for FDB, AQP4 was found to be significantly over-expressed in fast muscles, as reported by densitometric analysis (C); n = 6-8 per muscle; **p < 0.01 vs sed. D) Protein levels of AQP4 in QUAD muscles of four groups of treadmill runners based on their mean time run per day of both D10 and D30 animals. Significant changes in AQP4 protein levels were obtained after 30 min/day of endurance exercise; n = 7–11 per cluster. *p < 0.01 vs sed and <15' cluster. E–F) Effect of short-term exercise on AQP4 expression in QUAD muscles. Note that 5 days of endurance exercise (D5) did not determine a significant increase in AQP4 expression, in contrast with observations in D10 rats; n = 6–8 per group. *p < 0.01 vs sed. #p < 0.05 vs D5. In all the immunoblotting experiments, protein levels were corrected for whole protein loading determined by staining membrane with Ponceau S.

doi:10.1371/journal.pone.0058712.g001
AQP4 KO mice show impaired aerobic performance

Upregulation of AQP4 obtained in rats and mice subjected to endurance exercise suggests a physiological role of this water channel in skeletal muscle activity. We then decided to take advantage of the AQP4 KO murine model to evaluate the effect of the absence of AQP4 on skeletal muscle performance. As summarized in table 1, no change in body weight or muscle water percentage was found in AQP4 KO mice. However, EDL (-12.8%) and SOL (-17.4%) muscle weight were slightly reduced in AQP4 KO mice. Moreover, a slight but significant reduction in cross-sectional area (CSA) in both muscles was detected (EDL -15%; SOL -12%), suggesting that a slight muscle atrophy occurs in this AQP4 KO murine model. Even if atrophy was not the main aim of the present study, we measured the active phosphorylated protein content of Akt, (also called PKB; protein kinase B), a serine/threonine kinase, as a pivotal point in both hypertrophy and atrophy signaling pathways. As reported in fig. S2, we observed a significant increase in pAkt/Akt ratio of AQP4 KO mice compared to WT.

The impact of the AQP4 deletion in skeletal muscle function was then determined by subjecting AQP4 KO mice to an endurance treadmill exercise test. AQP4 KO mice showed about one third the running capacity of WT mice at D1 (WT 978.43 ±105.68 m vs AQP4 KO 363.8 ±93.77 m). Mice lacking AQP4 showed an improvement in daily distance at D10 (826.71 ±62.55 m) and D30 (735.85 ±27.46 m). However, when normalized to muscle weight, which was slightly lower in AQP4 KO than in WT (data not shown), the active tension development was not statistically different (fig. 5A, right panel). The time parameters of the twitch (time to peak, half rise and half relaxation times) did not show differences between gastrocnemius of WT and AQP4 KO mice (fig. 5B). Resistance to fatigue was evaluated by stimulating the muscle at 30 Hz for 0.5 s every second. The relative force decrease (fatigue index) after fatigue stimulation did not reveal any difference between WT and AQP4 KO muscles, suggesting a similar resistance to fatigue (fig. 5C).

Impaired osmotic water permeability in AQP4 KO skeletal muscle sarcolemma

The accumulation of AQP4 in fast muscle fibers after endurance training and the significant reduction of exercise tolerance in AQP4 KO mice ablation, which seems to be at odd with the preserved muscle contraction kinetics, prompted us to test the relevance of AQP4 for membrane permeability. To this end the osmotic behavior of skeletal muscle lacking AQP4 water channels was investigated by means of two different biophysical approaches: the TIRF microscopy on isolated muscle fibers [26], and the SFLS analysis on a highly enriched sarcolemma fraction, named light microsomes (LM) [27].

For TIRF-M experiments, small fiber bundles were mechanically isolated from WT and AQP4 KO EDL muscles and subjected to an osmotic challenge. Representative superimposed curves are shown in fig. 6A, in which the curve of WT fibers shows a faster osmotic response compared with that of KO fibers. The kinetic of osmotic volume changes was evaluated by comparing the time constants for swelling obtained from the
Figure 3. MHCs distribution in rat quadriceps muscles after treadmill activity. A) Representative immunofluorescence photomicrographs of MHC isoforms and AQP4 in quadriceps muscle from sed, D10 and D30 rats groups. Sections were immunostained for slow MHC, MHC IIA, and all MHC isoforms except MHC IIX. Scale bar, 50 μm. B) Percentage of fibers expressing MHC isoforms and AQP4 in rat muscles. Note that the distribution of MHCs and AQP4 did not change in either fast or slow-twitch muscles after D10 and D30 compared to sed rats (n = 5 muscles/group).

doi:10.1371/journal.pone.0058712.g003
AQP4 has a functional role in sarcolemma permeability of skeletal muscle fibers

Rapid changes in muscle cell volume occur in response to muscle contraction, which is associated with changes in hydrostatic forces, as well as intracellular generation of osmotically active solutes [1]. Thus, rapid water transport seems to have a physiological role in contraction-induced muscle swelling, and the presence of AQP4 water channels is consistent with this physiological need. However, the importance of AQP4 in skeletal muscle physiology has been questioned by the results obtained by others [16]. In a previous work [27] we pointed out that the biochemical technique employed to isolate sarcolemma vesicle is a crucial step for reliable functional studies conducted by SFLS. Indeed, water transport studies demonstrated high water permeability of purified LM vesicles of WT mice compared to those obtained from mdx mice, the murine model of DMD in which AQP4 decreased by about 90%.

The present results definitively demonstrate the functional role of AQP4 in water transport using SFLS analysis on LM vesicles purified from WT and AQP4 KO muscles and prepared according to Frigeri et al. [27]. Water transport studies demonstrated high water permeability of WT LM vesicles, which is consistent with AQP-mediated pathway for water movement. This value is about twofold that obtained in AQP4 KO LM, confirming the crucial role of the water channel to allow a quick response to changes in cellular osmolality. It is important to point out that AQP4 KO with CD1 genetic background used in our experiments is the same used by Yang and coworkers.

Osmotic water permeability was further analyzed by TIRF-M on intact muscle fibers from WT and AQP4 KO EDL muscles. Small bundles of few muscle fibers were dissected longwise, from tendon to tendon, with the use of microscissors and without using collagenase [28]. This preparation perfectly preserves the structure of muscle fibers and allows a correct measurement of water transport. The time constant from WT EDL fibers was approximately threefold smaller than that obtained from AQP4 KO EDL fibers. Our findings are in contrast with a previous study performed with spatial-filtering microscopy on segments of enzymatically isolated fiber bundles which showed that half times for osmotic equilibration were not affected by AQP4 deletion [16]. In our opinion, however, those measurements were likely affected by inappropriate fiber preparations (use of fibre segments,

Discussion

The major question addressed in this study is whether AQP4 plays a physiological role in determining skeletal muscle performance. To this purpose, four distinct approaches were adopted: endurance training on treadmill; free wheel running activity; analysis of in vivo and ex vivo contractile activity on hindlimb muscles; functional analysis of sarcolemma water permeability. The results support to the view that AQP4 is essential for long lasting contractile activity of fast skeletal muscles.

Table 1. Data are expressed as mean±SEM. *p<0.01, AQP4 KO vs WT.
enzymatic digestion, presence of unstirred layers) and were obtained using a technique different compared to TIRF-M.

Taken together, our results give strong evidence in favour of the functional role of AQP4 and shed new light on the involvement of this water channel in osmotic response of fast-twitch fibers in both normal and high activity conditions.

AQP4 in skeletal muscles is positively modulated during aerobic training

The Western blot analysis reported in this paper provides the first demonstration that exercise training induces AQP4 protein accumulation in fast-twitch muscles in proportion to the increased activity. The best rat runners (30–60 mins/day) were able to significantly increase AQP4 protein content compared to sed rats, suggesting that AQP4 up-regulation occurs when a threshold of daily activity is exceeded. The accumulation of AQP4 likely allows a rapid change of fibre volume and this is likely very important in relation to the large variations in intracapillary hydrostatic pressure and intracellular concentration of osmotically active molecules which occur during prolonged contractile activity (see [1] for a review). Indeed, this could better control or preserve intracellular osmolality. We did not observe any change in AQP4 expression in FDB or SOL muscles. The latter is a typical slow-twitch muscle, in which basal expression of the water channel is very low [26], while FDB muscle is an oxidative glycolytic fast-twitch muscle, with a peculiar low AQP4 expression in basal conditions in contrast with the high proportion of type II fibers (IIA). This confirm our previous conclusion that AQP4 expression is associated with the glycolytic capacity of the muscle. Functionally, the unaltered expression of AQP4 after exercise could be explained by the poor involvement of FDB muscle in running movements. Indeed, FDB has a postural function and, thus, it is weakly called on during exercise.

Figure 5. Treadmill and wheel running exercise in WT and AQP4 KO mice. A) Daily mean distance at D1, D10 and D30 of treadmill activity. Note that AQP4 KO mice ran significantly less than WT mice at D1 and throughout the training period; n = 6 per group. *p<0.05 and **p<0.01 vs the relative WT group. B) Progression of daily mean distance in WT and AQP4 KO mice; n = 24 per group. Note a two-phase behavior of AQP4 KO mice in treadmill exercise. C) Daily mean distance at D1, D10 and D30 of voluntary activity. AQP4 KO mice ran significantly less than WT mice during all the training period; n = 6 per group. **p<0.01 vs the relatives WT group. #p<0.01 vs D1 WT group. D) Progression of daily mean distance in WT and AQP4 KO mice; n = 24 per group.

dot:10.1371/journal.pone.0058712.g005
Long-term (>16 weeks) endurance exercise training of rodent muscles has been shown to induce MHCs-based fiber type transitions characterized by increase in type I and IIa fibers and corresponding decrease in the fastest type IIX and IIB fibers [29]. In our experiment, immunofluorescence analysis with anti-MHC antibodies did not reveal any change in fibre type distribution after 30 days of endurance training. This suggests that our protocol of 30 days of endurance exercise was not lasting enough to change MHC expression but was sufficient to increase AQP4 content without changing the number of fibers expressing AQP4. This finding suggests that AQP4 accumulation occurs in a fiber-type specific manner, probably in relation to specific metabolic adaptations of fast-twitch fiber muscles to exercise. In fact, slow and fast muscles differ in the relative role of glycolysis and oxidative phosphorylation. Slow muscles are able to generate all ATP they need by oxidative mitochondrial processes; actually, their ATP consumption during contraction is not that high, and this contributes to their ability to maintain contractile activity for long time without showing fatigue. Fast muscles rely upon glycolytic processes to generate ATP very rapidly, and this sets a limit to the duration of their contractile activity. The selective increase in AQP4 expression in fast-twitch fibers should confer the ability to protect contraction activity by changing quickly the volume, in order to preserve a constant osmolarity when large amount of lactate and P_i are generated during intense contractile activity.

Interestingly, AQP4 protein level increases in rat muscles without changes in total mRNA copy number. This finding suggests that post-transcriptional regulation mechanisms may be involved. In particular, as recently reported [30], translational regulation of AQP4-M1 mRNA via Leaky Scanning and Reinitiation mechanisms, associated to an out-of-frame uORF, is able to modulate the M1/M23 ratio and AQP4 protein abundance. Many different types of cell stress are able to modify the re-initiation efficiency [31]. It is tempting to speculate that endurance exercise may increase the re-initiation efficiency, resulting in the increase in protein abundance leaving the mRNA copy number unaltered. However, we can exclude any involvement of stress conditioning induced by exercise in muscle AQP4 increase during prolonged activity, as demonstrated by results obtained in SS5 and SS10 rats muscles.

Aerobic performance is decreased by AQP4 ablation without impairment of skeletal muscle contraction

The effects of training on AQP4 accumulation were consistently found in rats and in WT mice thus providing a direct reference for the studies on AQP4 KO mice. Actually, AQP4 KO mice allowed to perform voluntary exercise on the wheel or subjected to forced training on the treadmill showed less activity than WT mice.
during the entire observation period. We would like to highlight that significant improvement of performances occurred in WT and AQP4 KO from D1 to D30 in both the paradigms. This result suggests that the absence of AQP4 does not prevent physical activity but sets a limit to reaching the same performance achieved by WT muscles. Importantly, the absence of AQP4 did not affect muscle structure in terms of fiber type distribution or myofibrillar organization. Indeed, we never observed differences between WT and AQP4 KO mice in contractile properties, fatigue resistance or force generation in fast-twitch muscles analysed \textit{in vivo} (gastrocnemius) and \textit{ex vivo} (EDL), in full accordance with other studies [16]. However, AQP4 KO mice appeared to be less able to perform sustained activities, as required in running exercise. These findings suggest that when a significant metabolic effort is necessary, AQP4 expression becomes important or even essential. Considering that AQP4 accumulation was observed after ten days of exercise, its involvement in mechanisms underlying skeletal muscle fatigue likely occurs after several bouts of activity. Thus, the absence of differences observed in \textit{in vivo} and \textit{ex vivo} activities could be attributed to the short duration of the contractile activity, which may not require the activation of AQP4-dependent regulatory mechanisms.

Our data reveal slight muscle atrophy in AQP4 KO mice. Since reduction of muscle mass also occurs in soleus, which expresses very low levels of AQP4, we can conclude that muscle atrophy is not directly related to the absence of AQP4 in muscle fibers, but perhaps to a lower AQP4 null mouse spontaneous physical activity. In order to further confirm alteration of the mechanisms underlying muscle mass dynamics we analysed the levels of active Akt in AQP4 null mice. Surprisingly, Akt was more phosphorylated in AQP4 KO than in WT mice, further supporting an alteration of signaling pathway that regulates both protein synthesis and degradation. Interestingly, a recent study demonstrates elevated Akt expression and Ser(473) phosphorylation associated to muscle atrophy in a mouse model of Huntington's [32]. A more detailed analysis on Akt atrophy regulatory targets, including Foxo1, Foxo3, atrogin-1 and MuRF1, as well as its downstream hypertrophy signaling targets, such as GSK-3β, mTOR, p70S6K and 4E-BP1, is required in order to precisely identify the signaling pathway activated in absence of AQP4, but falls out of the scope of the present study.

Taken together the results obtained in the present study point to a crucial role of AQP4 in determining aerobic performance of fast skeletal muscles. Ablation of AQP4 implies reduced membrane permeability and this likely impairs the ability to perform long lasting exercises, even if the contraction machinery is well preserved. Muscle atrophy (this study) and reduced expression of metabolic enzymes [15] follow the decrease in spontaneous activity in mice lacking AQP4. Endurance training can increase aerobic performance also in mice carrying null mutation of AQP4. Those mice, however, never reach the same performance of WT mice, thus suggesting that the accumulation of AQP4 is essential to improve the aerobic performance. Moreover, preliminary data revealed a dramatic alteration of glycolytic pathway coupled with perturbed calcium homeostasis in absence of AQP4 (unpublished preliminary data), supporting the hypothesis that this protein

Figure 7. Force generation by WT and AQP4 KO EDL muscles \textit{“ex vivo”}. A) Twitch tension (n = 12). B) Maximal tetanic tension (n = 12). C) Twitch times (n = 12). D) Fatigue index (n = 12). In each case, no difference was found between WT and AQP4 KO muscles.
doi:10.1371/journal.pone.0058712.g007
channel is (directly or indirectly) involved in cellular mechanisms important for energy supply.

In conclusion, we show here for the first time that prolonged endurance training promotes AQP4 accumulation in skeletal muscle, leading to improved exercise tolerance without changing fiber type. This study demonstrates the important function of the water channel in skeletal muscle physiology, showing that AQP4 removal has a dramatic impact on osmotic water permeability and mouse physical activity. AQP4 KO mice showed significant attenuation of mean distances covered in both voluntary and forced activities, even though contractile properties measured by ex vivo and in vivo tests were unvaried when compared to WT mice.

These findings confirm that AQP4 plays a pivotal role in the high water permeability of the plasmalemma of fast-twitch fibers, and during adaptive processes that confer the metabolic phenotype leading to improved fatigue resistance during prolonged exercise. This study brings further support to the physiological role of the water channel in sustaining muscle contractile activity and in the regulation of metabolic changes occurring during exercise.

Further studies are required to evaluate if other components, such as ion handling machinery and atrophy-related pathways, are altered in skeletal muscle of AQP4 KO mice and may contribute to the observed phenomena.

Materials and Methods

Ethics statements

All experiments conformed to international guidelines on the ethical use of animals and were designed to minimize the number of animals used and their suffering. Experiments in this study were approved by the Italian Health Department (Art. 9 del Decreto Legislativo 116/92).

Animals

6 weeks-old male Wistar rats (weight 125–150 g) were used in the experiments. AQP4 KO mice with a CD1 genetic background were kindly provided by Dr. Hu (Nanjing Medical University, China). The generation of this AQP4 KO mice model has been
previously reported [33]. The mice used here were bred and genotyped in the approved facility at the University of Bari. Mice were kept on a 12 h light-dark cycle with food and water ad libitum. Male WT and AQP4−/− mice with a CD1 genetic background, aged 3 months, were used in these experiments.

**Treadmill exercise**

A stress-free endurance treadmill exercise running protocol was adopted [34] with some modifications between rats and mice. Rats were randomly assigned to D5 (5 days of exercise; n = 10), D10 (n = 25) and D30 (n = 10) groups. In brief, after three days of treadmill familiarization at a speed of 0.8 kmph for 5 min to eliminate novelty and stress effects, animals ran on a custom-made motor-driven treadmill at a starting speed of 0.8 kmph, increasing the speed by 0.1 kmph every 2 mins until the maximum speed of 2 kmph (about 34 m/min) until exhaustion. In the mice treadmill exercise protocol, AQP4 KO and WT mice were randomly assigned to D10 and D30 groups. The maximum speed was set at 1.4 kmph (about 24 m/min), whereas the other parameters were unvaried.

**Voluntary wheel running exercise**

Age-matched WT and AQP4 KO mice were randomly assigned either to D10 (n = 12) or D30 (n = 12) groups and individually placed in cages with free access to a running wheel equipped with a cyclocomputer (SigmaSport, Germany), whereas animals in the control group (sed) were housed in cages without the wheel. Daily mean distances were registered throughout the period of the experiment.

**Sample preparation and Western Blotting**

Muscle samples were prepared as previously described [7] with some modifications. On the basis of the size of muscles, several 10-μm-thick cryosections of skeletal muscle were dissolved in 150 μl of RIPA lysis buffer (10 mM Tris–HCl pH 7.4, 140 mM NaCl, 1% n-dodecyl-β-D-maltoside, 1% Na-deoxycholate, 0.1% SDS, 1 mg/ml PMSF, 1X Protease Inhibitor Cocktail (Roche Bioscience). Water permeability was measured using a Nikon round glass coverslip previously treated with Cell Tak (BD Bioscience). Images were analyzed using Scion Image software. For relative quantification, the optical density value was determined for equal sized boxes drawn around antibody-stained bands, with background values taken below each band of interest to account for non-specific antibody staining in the lane. Normalization was performed using reversible Ponceau S staining, according to Romero-Calvo et al. [22].

**Densitometry**

Images were analyzed using Scion Image software. For relative quantification, the optical density value was determined for equal sized boxes drawn around antibody-stained bands, with background values taken below each band of interest to account for non-specific antibody staining in the lane. Normalization was performed using reversible Ponceau S staining, according to Romero-Calvo et al. [22].

**Immunofluorescence analysis**

8-μm transverse sections were prepared using a cryostat (CM 1900; Leica, Germany), collected from the midpoint of each muscle at -20°C and stored on positively charged glass slides (Thermo Scientific). Sections were acclimated to RT for 15 min, fixed with 4% para-formaldehyde (Sigma, Milan, Italy) for 10 min, and blocked using 0.1% gelatin diluted in PBS for 30 min at RT. Sections were then incubated at 37°C for 1 h with monoclonal antibodies directed against AQP4 (dilution 1:300) and adult MHC isoforms [35] harvested from hybridoma cell lines (Developmental Hybridoma Studies Iowa, USA; BA-D5 (anti-MHC I; dilution 1:300), SC-71 (anti-MHC IIA; dilution 1:500), and BF-35 (all MHCs, but not MHC IIX; dilution 1:1000). Primary antibodies were detected by AlexaFluor 488 anti-goat and 594 anti-mouse (Life Technologies, USA) secondary antibodies diluted at 1:1000. Secondary antibodies were incubated for 1 h at RT.

**RNA extraction and Real Time PCR mRNAs analysis**

The analysis of AQP4-mRNA levels was performed by Real-Time qPCR, using an absolute quantification approach [36]. RNA extraction from rat quadricepses was carried out using Trizol Reagent (Life Technologies, USA) in according with manual instruction. 5 μg of total RNA was retro-transcribed with 100 ng of random primers using Super Script III (Life Technologies, USA) and the cDNA was Real-Time PCR amplified using SybrGreen Chemistry (Life Technologies, USA) and the primers indicated in brackets (forward: CGGTTCATGGAAAACCTC-CACT; reverse: CATGCTGGCTCCGGGTATAAT), which are able of amplifying the Mz, M1 and M23 mRNAs. The standard curve approach was used to obtain absolute quantification of the AQP4 mRNAs (Mz+M1+M23). The standard curve for AQP4-Mz+M1+M23 mRNAs quantification was obtained using pcDNA3.1 (circular or linear) containing the rat AQP4-M23 CDS.

**Isolation of fibers and TIRF measurements**

For the swelling assay by TIRF, small bundles of 5–10 EDL muscle fibers from WT and AQP4 KO mice arranged in a single layer were dissected longwise, tendon to tendon, with the use of microscissors and without enzymatic digestion [28]. Muscle fibers were incubated with the fluorescent dye chloromethyl-benzoyl-amino-tetramethyl-rhodamine (CM-TMR - Life Technologies, USA) to a final concentration of 10 μM for 30 min at RT in normal physiological (NP) solution [28]. After they were loaded and washed, muscle fibers were attached onto 20 mm-diameter round glass coverslip previously treated with Cell Tak (BD Bioscience). Water permeability was measured using a Nikon Laser TIRF setup as described by Psani et al. [37]. Fibers were initially perfused with isotonic NP solution (30 mL/min, temperature 10°C) and then subjected to hypoosmotic treatment by reducing the NaCl concentration of NP solution. The time course of TIR fluorescence, measured in response to osmotic gradient, was used to assess the osmotic properties of skeletal muscle fibers. The kinetics of osmotic volume changes was characterized by...
determined according to the following equation:

\[
\mathcal{P}_f = k \left[ V_w \times \frac{S}{V} \right] \Delta \text{osm}
\]

where \( V_w \) is the partial molar volume of water (18 cm\(^3\)/mol), \( S/V \) is the ratio of the vesicle surface area to the initial volume, and \( \Delta \text{osm} \) is the osmotic difference between the initial intra- and extra-vesicular sucrose concentration.

Ex-vivo muscle mechanics

EDL muscles were dissected from mice killed by cervical dislocation placed in warm oxygenated Krebs solution and mounted between a force transducer (AME-801 SensorOne, Sausalito, California) and a micro-manipulator controlled shaft in a small chamber where oxygenated Krebs solution was continuously circulating. Temperature was kept constant at 25°C. The stimulation conditions were optimized and muscle length was increased until force development during tetanus was maximal. The responses to a single stimulus (twist) or to a series of stimuli at various rates producing unfused or fused tetani were recorded. Time to peak tension, time to half relaxation and peak tension were measured in single twitches. Tension was measured in completely fused maximal tetani and the twitch/tetanus ratio was determined. The resistance to fatigue was tested by stimulating the muscles with a fatiguing protocol based on 0.5 s fused tetani with a 1:4 duty ratio (low frequency fatigue).

Statistics

For protein levels analysis, AQP4 mRNA copy number analysis, voluntary and endurance exercises, differences were determined by One-way ANalysis Of VAriance (ANOVA) followed by Tukey’s post hoc multiple comparison test. For TIRF-M, SFLS, ex-vivo and in vivo activity the paired t-test was used to compare means of time and tension parameters. Data were presented as means ± SEM except that clusters of running time performances were represented as median ± quartiles. Significance was set at p<0.05.

Supporting Information

Figure S1 A) Protein loading determination by Ponceau S staining of PVDF membranes of rat muscles samples. B) Assessment of the specificity of AQP4 antibody by immunoblotting analysis. Lanes 1 to 4 are transfected V79 cells. Lane 1 is pTarget-AQP4-M1wt, lane 2 is pTarget-AQP4-M23wt, lane 3 is untransfected V79 cells, and lane 4 is empty pTarget. Identical-sized control brain lysate (lane 7) and LM (lane 5) from WT mice. Lane 6 is LM from AQP4 KO mice (lane 6). (TIF)

Figure S2 A) Immunoblotting analysis of ser473-phosphorylated Akt (pAkt) and total Akt in skeletal muscle of WT and AQP4 KO mice. B) Densitometric quantification of pAkt/Akt ratio (n = 4–5) revealed a significant increase in activated Akt from muscles lacking AQP4. **p<0.01 vs WT. (TIF)

Acknowledgments

It is a pleasure to acknowledge the conscientious effort of Mr. Gaetano de Vito in maintaining healthy rodent colonies during all the phases of the experiments. We are also grateful to Stefania Rosito, Annalisa Scarcelli, Nunzia Panzini and Giuseppina Mastrototaro for their helpful technical assistance. We would also thank Dr. Hu (Nanjing Medical University, China) for providing AQP4 KO mice.

BA-D5, SC-71 and BF-35 monoclonal antibodies developed by Prof. Stefano Schiaffino were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

Author Contributions

Conceived and designed the experiments: DB BB CR AF. Performed the experiments: DB BB FP AS GPN MGM. Analyzed the data: DB BB FP AS
CR AF. Contributed reagents/materials/analysis tools: CR MS AF. Wrote the paper: DB BF CR MS AF.

References

1. Usher-Smith JA, Huang CL, Fraser JA (2009) Control of cell volume in skeletal muscle. Biol Rev Camb Philos Soc 84: 143–159.
2. Frigeri A, Gropper MA, Umenishi F, Kawashima M, Brown D, et al. (1995) Localization of MIVWC and GLIP water channel homologs in neuromuscular, epithelial and glandular tissues. J Cell Sci 108 ( Pt 9): 2993–3002.
3. Jung JS, Bhat RV, Preston GM, Guggino WB, Barabam JM, et al. (1994) Molecular characterization of an aquaporin cDNA from brain; candidate osmoreceptor and regulator of water balance. Proc Natl Acad Sci U S A 91: 13032–13036.
4. Verbavatz JM, Ma T, Gobin R, Verkman AS (1997) Absence of orthogonal arrays in kidney, brain and muscle from transgenic knockout mice lacking water channel aquaporin-4. J Cell Sci 110 ( Pt 22): 2855–2860.
5. Silberstein C, Bouley R, Huang Y, Fang P, Pastor-Soler N, et al. (2004) Membrane organization and function of M1 and M25 isoforms of aquaporin-4 in epithelial cells. Am J Physiol Renal Physiol 287: F501–511.
6. Nicchia GP, Rossi A, Mola MG, Pisani F, Stigliano C, et al. (2010) Higher order structure of aquaporin-4. Neuroscience 168: 903–914.
7. Ascereto S, Mastrotoraro M, Stringara S, Giazzotto E, Beoda P, et al. (2008) Aquaporin-4 expression is severely reduced in human sarcoglycanopathies and dysferlinopathies. Cell Cycle 7: 2199–2207.
8. Frigeri A, Nicchia GP, Nico B, Quondamatteo F, Herken R, et al. (2001) Aquaporin-4 deficiency in skeletal muscle and brain of dystrophic mdx mice. Faseb J 15: 90–98.
9. Frigeri A, Nicchia GP, Repetto S, Bado M, Minetti C, et al. (2002) Altered aquaporin-4 expression in human muscular dystrophies: a common feature? Faseb J 16: 1120–1122.
10. Schiaffino S, Reggiani C (2011) Fiber types in mammalian skeletal muscles. Physiological reviews 91: 1447–1531.
11. Wakayama Y, Jimi T, Inoue M, Kojima H, Murahashi M, et al. (2002) Reduced plasma volume and ion regulation during exercise after low- and high-carbohydrate diets. Am J Physiol 266: R1896–1906.
12. Greenleaf JE, Van Beaumont W, Brock PJ, Morse JT, Mangseth GR (1979) Analysis by two-dimensional Blue Native/SDS-PAGE of membrane protein arrays in kidney, brain and skeletal muscle. J Physiol 285: 111–115.
13. Kaakinen M, Salmela P, Zelenin S, Metsikko K (2007) Distribution of aquaporin-4 on sarcolemma of fast-twitch skeletal myofibres. Cell Tissue Res 329: 529–539.
14. Verbavatz JM, Ma T, Gobin R, Verkman AS (1997) Absence of orthogonal arrays in kidney, brain and muscle from transgenic knockout mice lacking water channel aquaporin-4. J Cell Sci 110 ( Pt 22): 2855–2860.
15. Basco D, Nicchia GP, Desaphy JF, Camerino DC, Frigeri A, et al. (2010) Reversible Ponceau staining as a loading control alternative to actin in Western blots. Analytical biochemistry 401: 318–320.
16. Aldridge GM, Podrebarac DM, Greenough WT, Weiler IJ (2000) The use of total protein stains as loading controls: an alternative to high-abundance single-protein controls in semi-quantitative immunoblotting. Journal of neuroscience methods 172: 250–254.
17. Chabrier G, Dittmer J (2006) Beta-actin is not a reliable loading control in Western blot analysis. Electrophoresis 27: 2844–2845.
18. Frigeri A, Nicchia GP, Bado M, Minetti C, Beoda P, et al. (2008) Aquaporin-4 expression is severely reduced in human sarcoglycanopathies and dysferlinopathies. Cell Cycle 7: 2199–2207.
19. Frigeri A, Nicchia GP, Verbavatz JM, Valenti G, Svelto M (1998) Expression of aquaporin-4 in fast-twitch fibers of mammalian skeletal muscle. J Clin Invest 102: 695–703.
20. Frigeri A, Nicchia GP, Balena R, Nico B, Svelto M (2004) Aquaporins in skeletal muscle: reassessment of the functional role of aquaporin-4. Faseb J 18: 905–907.
21. Hinnebusch A (2000) In:Sonenberg N, Hershey JWB, Mathews MB, editors. Translational Control of Gene Expression. Cold Spring HarborvNY :Cold Spring Harbor Laboratory Press. pp. 185–243.
22. Kaakinen M, Salmela P, Zelenin S, Metsikko K (2007) Distribution of aquaporin-4 on sarcolemma of fast-twitch skeletal myofibres. Cell Tissue Res 329: 529–539.
23. Hinnebusch A (2000) In:Sonenberg N, Hershey JWB, Mathews MB, editors. Translational Control of Gene Expression. Cold Spring HarborvNY :Cold Spring Harbor Laboratory Press. pp. 185–243.
24. Bee P, Zhang Z, Marchionni D, Diaz WC, Jetton TJ, et al. (2011) Molecular characterization of skeletal muscle atrophy in the R6/2 mouse model of Huntington's disease. American journal of physiology Endocrinology and metabolism 301: E49–61.
25. Frayssie B, Desaphy JF, Piccini S, De Luca A, Lantion A, et al. (2003) Decrease in resting calcium and calcium entry associated with slow-to-fast transition in unloaded rat soleus muscle. Faseb J 17: 1916–1918.
26. Schiaffino S, Reggiani C (2011) Fiber types in mammalian skeletal muscles. Physiological reviews 91: 1447–1531.
27. Schiaffino S, Reggiani C (2011) Fiber types in mammalian skeletal muscles. Physiological reviews 91: 1447–1531.
28. Pisani F, Rossi A, Nicchia GP, Svelto M, Frigeri A (2011) A Translational regulation mechanisms of aquaporin-4 supramolecular organization in astrocytes. Glia 59: 1923–1932.
29. Frohlich JF, Desaphy JF, Camerino DC, Frigeri A, et al. (2010) Analysis by two-dimensional Blue Native/SDS-PAGE of membrane protein alterations in rat soleus muscle after hindlimb unloading. Eur J Appl Physiol 110: 1213–1224.
30. Frigeri A, Nicchia GP, Desaphy JF, Piccini S, De Luca A, et al. (2001) Muscle loading modulates aquaporin-4 expression in skeletal muscle. Faseb J 15: 1282–1289.