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Activation of osmolyte pathways in inflammatory myopathy and Duchenne muscular dystrophy points to osmoregulation as a contributing pathogenic mechanism

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Alongside well-known nuclear factor κB (NFκB) and its associated cytokine networks, nuclear factor of activated T cells 5 (NFAT5), the master regulator of cellular osmoprotective programs, comes forward as an inflammatory regulator. To gain insight into its yet unexplored role in muscle disease, we studied the expression of NFAT5 target proteins involved in osmolyte accumulation: aldose reductase (AR), taurine transporter (TauT), and sodium myo-inositol co-transporter (SMIT). We analyzed idiopathic inflammatory myopathy and Duchenne muscular dystrophy muscle biopsies and myotubes in culture, using immunohistochemistry, immunofluorescence, and western blotting. We report that the level of constitutive AR was upregulated in patients, most strongly so in Duchenne muscular dystrophy. TauT and SMIT expression levels were induced in patients' muscle fibers, mostly representing regenerating and atrophic fibers. In dermatomyositis, strong staining for AR, TauT, and SMIT in atrophic perifascicular fibers was accompanied by staining for other molecular NFAT5 targets, including chaperones, chemokines, and inducible nitric oxide synthase. In these fibers, NFAT5 and NFκB p65 staining coincided, linking both transcription factors with this important pathogenic hallmark. In sporadic inclusion body myositis, SMIT localized to inclusions inside muscle fibers. In addition, SMIT was expressed by a substantial subset of muscle-infiltrating macrophages and T cells in patient biopsies. Our results indicate that osmolyte pathways may contribute to normal muscle functioning, and that activation of AR, TauT, and SMIT in muscle inflammation possibly contributes to the tissue's failing program of damage control.
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Chronic infiltration of skeletal muscle by immune cells can be observed in different diseases. In the idiopathic inflammatory myopathies (IIM), which include dermatomyositis (DM), polymyositis (PM), sporadic inclusion body myositis (IBM), and necrotizing autoimmune myopathy (NAM), it originates from an autoimmune response.[1] In hereditary muscle diseases, such as Duchenne muscular dystrophy (DMD), inflammation results from continuous tissue damage. It is commonly accepted that nuclear factor κB (NFκB) has a pivotal role in the IIM,[2] and for DMD we also showed its activation.[3] Acting as an inflammatory stimulator, this transcription factor induces the expression of tissue adhesion molecules and a plethora of cytokines, initiating and amplifying tissue inflammation. Our former results, showing lymphotoxin β (LTβ) expression as an early event in muscle inflammation,[4] lead us to investigate also the LTβ-inducer nuclear factor of activated T cells 5 (NFAT5), which we found to be constitutively expressed in healthy skeletal muscle. Mitogen-activated protein kinase p38-mediated activation of NFAT5 switches on an osmoprotective repertoire in response to hypertonic conditions. Similar to many transcription factors, NFAT5 can be controlled at multiple levels including increased expression, nuclear translocation, and/or increased activity through post-translational modifications.

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Pro-inflammatory stimuli, such as TNFα and IL-1β, have been shown to activate NFAT5,[5] offering an important link between NFκB and NFAT5 transcription factor pathways.

NFAT5 is first and foremost thé central regulator of the cell's response to osmotic stress. Hypertonicity leads to the osmotic efflux of water from the cells, and subsequent cell shrinkage. Cells restore their volume and normalize intracellular salt concentration by accumulating osmotically active yet non-perturbing organic solutes, among which are the polyols sorbitol and myo-inositol and the amino acid taurine. Sorbitol is primarily synthesized intracellularly from glucose through a reaction catalyzed by aldose reductase (AR; *AKR1B1*, MIM103880). Taurine and myo-inositol accumulate within the cells due mostly to an increased import from the extracellular fluid by transporter-mediated uptake, more particularly by the solute carrier family proteins (SLC) taurine transporter (TauT; *SLC6A6*, MIM186854) and sodium myo-inositol co-transporter (SMIT; *SLC5A3*, MIM600444). SMIT is a plasma membrane protein that uses the electrochemical sodium gradient across the membrane to import myo-inositol into the cell.[6] Osmolyte pathways are highly regulated by cellular osmolarity, illustrated by the hypertonicity-induced increase of AR, TauT, and SMIT activity in kidney tissue, which results from increased gene transcription.[7]

As NFAT5 is present in skeletal muscle tissue but is further activated in inflammatory muscle disease, we investigated the transcription factor's downstream osmolyte pathways. AR, TauT, and SMIT expression levels were studied in muscle tissues from IIM and DMD patients and in myotubes in culture. We show upregulation in patients, which is in line with the exciting novel views linking adaptive immunity with hyperosmolarity. [8,9]

MATERIALS AND METHODS

Patients and Cell Lines

Patient material was collected at the University Hospitals of Ghent, Antwerp, Barcelona and Aachen. Frozen diagnostic limb muscle biopsies were obtained after informed patient and/or parent consent, and the methodologies used were reviewed and approved by the hospitals' Local Ethics Committees. Patient data are listed in Supplementary Tables S1 and S2. The *DMD* gene of patients ($n=6$) was sequenced to reveal duplications, deletions, insertions, and point mutations. Diagnosis of IIM was based on conventional clinical and myopathological criteria.[10–12] The diagnosis of PM was made only when non-necrotic invaded muscle fibers were present in the diagnostic biopsy and when patients had subsequently reacted to immunosuppressive therapy. IIM samples were from adult ($n=13$) and juvenile ($n=5$) DM, PM ($n=8$), IBM ($n=11$), and statin-induced NAM ($n=4$) patients. None of the DMD and IIM patients received immunosuppressive therapy before the biopsy, and all patients had progressive disease at the time of biopsy. Muscle specimens from disease controls with polyneuropathy were tested, muscle from subjects with no clinical, electromyographic, or histological evidence of myopathy served as healthy controls ($n=20$).

CCL-136 rhabdomyosarcoma and 5-8211 DMD cell lines were obtained from the Banque de cellules et d'ADN (Généthon, Paris, France). Cells were grown in Dulbecco's Modified Eagle Medium, supplemented with penicillin (50 U/ml), streptomycin (50 mg/ml; Gibco, Invitrogen, Carlsbad, USA), 10% fetal calf serum (Cambrex, Bioscience, Walkersville, USA), and 0.5% chick embryo extract (Accurate, Westbury, USA). Myoblasts differentiated into myotubes in culture medium containing 2% heat-inactivated horse serum for 48 h. Cells were maintained at 37 °C in a 5% CO_2 incubator. To evaluate the effects of hyperosmotic conditions on myotubes, up to 100 mM of NaCl was added to the culture medium for 24 h.

Immunohistochemistry and Immunofluorescence

Six-micrometer cryostat sections were cut from frozen muscle biopsies. Sections were treated with blocking solution containing 5% donkey serum, 10% heat-inactivated human serum, and 2% bovine serum albumin in phosphate-buffered saline. Incubations with primary antibodies were carried out in the same solution. The commercial antibodies that were used for immunolocalization are listed in Supplementary Table S3. Indirect immunohistochemical staining was carried out using the labeled streptavidin–biotin kit (LSAB; Dako, Glostrup, Denmark) visualized with 3,3′-diaminobenzidine (Dako). Cell nuclei were counterstained with hematoxylin (Gill N°2, Sigma-Aldrich, St Louis, MI, USA) and mounted with aquatex (Merck, Darmstadt, Germany). For fluorescent detection, secondary antibodies used were labeled with CY3 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and AlexaFluor488 (Invitrogen, Carlsbad, CA, USA). To allow double staining with mouse monoclonals, FITC-labeled anti-CD68 (Dako) was used. Slides were mounted with Fluoromount (Southern Biotech, Birmingham, AL, USA) and analyzed under a fluorescence microscope (Zeiss, Goettingen, Germany). Conventional semiquantitative scoring of staining intensity was performed by two non-blinded independent observers. Negative control studies consisted of the omission of primary antibody and the substitution by non-immune IgGs. Positive control tissues for immunostaining were frozen sections from breast tissue (NFAT5), Jurkat cells (AR), HeLa cells (TauT), and frozen sections containing kidney medulla (SMIT). Blocking studies were performed, and it was shown that 50 μg/ml (NFAT5; SP5110CP, Acris, San Diego, CA, USA) and 20 μg/ml (SMIT; sc-23142P, Santa Cruz Biotechnology, Santa Cruz, CA, USA) of peptide completely abrogated the immunofluorescent signal. Blocking peptides were not available for AR and TauT procedures.

Western Blotting

Total protein extracts were prepared by homogenizing pelleted cultured cells or frozen muscle samples in 2 volumes

of extraction buffer (50 mM TrisHCl, 2 mM EDTA pH 7.4) supplemented with protease inhibitors (TM mini protease inhibitor cocktail; Roche, Indianapolis, IN, USA). Fractionated cytoplasmic/nuclear extracts were prepared using the NE-PER Kit (Pierce, Rockford, IL, USA), following the manufacturer's instructions. To pellet debris, samples were centrifuged at 2000 *g* for 10 min. Samples were prepared for electrophoresis by adding lithium dodecyl sulfate sample buffer and a reducing agent (Invitrogen). Samples were boiled for 3 min and loaded onto 10% bis-tris gels. Proteins were transferred to nitrocellulose membranes by electroblotting, and protein bands were visualized using the chromogenic Western Breeze kit (Invitrogen). Densities of protein bands were calculated with the Quantity One software (Bio-Rad, Hercules, CA, USA), using glyceraldehyde-3-phosphate dehydrogenase or actin levels as internal standards. Results are given as mean ± s.d. For statistical analysis, the Student's *t*-test was performed; *P*-values below 0.05 were considered statistically significant.

RESULTS

Immunolocalization Studies

Results of AR, TauT, and SMIT immunolocalization in the skeletal muscle are summarized in Table 1 and are graphically represented in Supplementary Figure S1.

Normal muscle and disease controls

In normal skeletal muscle tissues, NFAT5 expression was prominent in myonuclei, with accentuation of the nuclear membrane, and was more discrete and variable on the muscle fiber membranes. Ser 1197-phosphorylated NFAT5 could rarely be shown in healthy controls, with muscle fibers staining negative to slightly positive in the sarcoplasm. Basal low sarcoplasmic levels of AR were detected in normal muscle tissues. In muscle biopsies displaying neurogenic atrophy, CD56+ fibers had variable AR staining intensities, with the smaller CD56+ fibers staining faintly and the larger fibers displaying higher levels of AR. In normal tissues, muscle fibers were TauT- and SMIT-negative. Part of blood vessels were AR- and SMIT-positive, but vascular TauT staining could not be observed (data not shown).

Dermatomyositis

NFAT5 staining was present on the membranes of myonuclei in virtually all muscle fibers, as confirmed with DAPI triple stains, whereas sarcoplasmic expression was mostly restricted to the perifascicular atrophic fibers (Figure 1a). Staining with a phospho-specific antibody showed that the sarcoplasmic signal corresponded to NFAT5 phosphorylated on its Serine 1197 residue (Figure 1b). The basal levels of AR and of heat shock protein 70 family chaperones (HSP70), that could be detected in the sarcoplasm of controls, were strongly increased in the perifascicular atrophic muscle fibers (Figure 1c and d), part of which were CD56+ (Figure 1e). In the scattered small fibers, AR upregulation was less pronounced. The sarcoplasm of part of the perifascicular atrophic fibers was TauT-positive (Figure 1f), of which most were CD56+ (Figure 1e). SMIT was upregulated in the perifascicular and scattered atrophic fibers, displaying combined myonuclear, membranous, and sarcoplasmic staining patterns. Blood vessels were mostly NFAT5- (Figure 1a and b), AR-, and TauT-negative; part of arterioles were SMIT-positive. AR and TauT could not be detected in inflammatory cells, whereas the majority of CD68+ macrophages and part of regenerative

Table 1 Immunolocalization of AR, TauT, and SMIT in muscle tissues

Diagnosis	Tissue constituent	AR	TauT	SMIT
Normal	CD56− MF	+	−	−
	Capillaries	−/+	−	−/+
	Arterioles	−/+	−	−
DM	Perifascicular atrophic MF	++	++	++
	Scattered CD56+ MF	+/++	++/+	++/+
	CD56− MF	+	−	+
	Capillaries	−/+	−	+/−
	Arterioles	−/+	−	−/+
	T cells	−	−	−/+
	Macrophages	−	−	+/−
	B cells	−	−	−
PM/IBM	Scattered CD56+ MF	+	−/+	++
	Non-necrotic invaded MF	+	−	+/++
	p62+ inclusions in MF	+	−	+
	CD56− MF	+	−	+
	Capillaries	−/+	−	−/+
	Arterioles	−/+	−	−
	T cells	−	−	+/−
	Macrophages	−	−	+/−
NAM	Scattered CD56+ MF	+/++	−/+	−/+
	CD65− MF	+	−	−
	T cells	−	−	−
	Macrophages	−	−	−
DMD	Scattered CD56+ MF	+	++	++
	Other CD56− MF	++/+	−	+
	Capillaries	−/+	−	+/−
	Arterioles	−/+	−	+/−
	T cells	−	−	−/+
	Macrophages	−	−	+/−

Abbreviations: AR, aldose reductase; DM, dermatomyositis; DMD, Duchenne muscular dystrophy; IBM, sporadic inclusion body myositis; MF, muscle fiber; NAM, necrotizing autoimmune myopathy; PM, polymyositis, SMIT, sodium myo-inositol co-transporter; TauT, taurine transporter.
Tissue constituents were scored: (−) negative; (+) positive; and (++) strongly positive. Most frequent staining pattern is indicated before /.

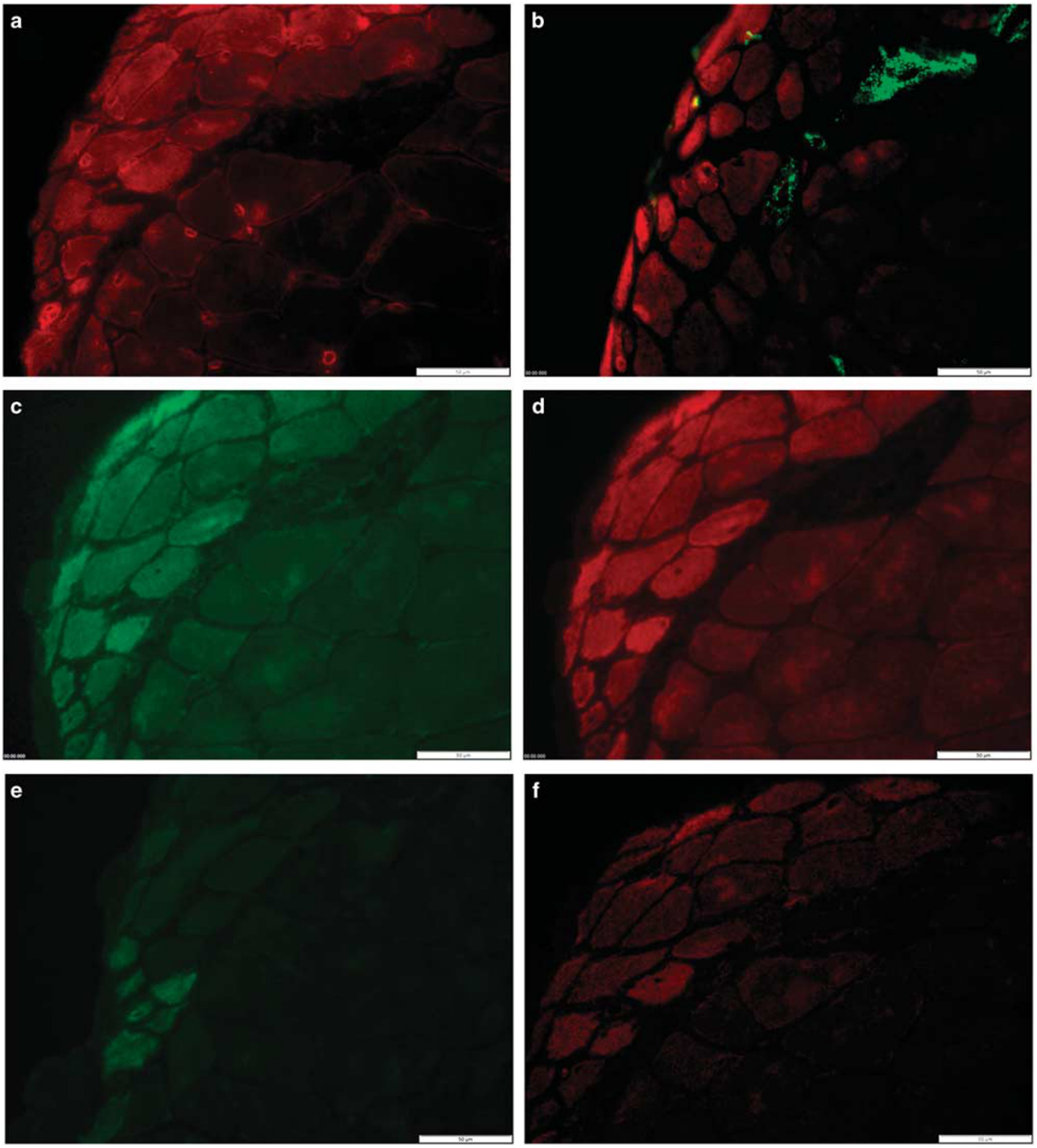


Figure 1 The osmolyte pathway and associated factors in juvenile dermatomyositis. Sequential immunofluorescent staining is shown in non-consecutive sections of a zone with perifascicular muscle fiber atrophy in the biopsy of patient DM17. (**a**) On the muscle fiber membrane and myonuclear membranes, NFAT5 (CY3, red) staining is present throughout, accompanied by sarcoplasmic staining only in the perifascicular atrophic fibers. (**b**) Staining with a specific antibody shows that the sarcoplasmic component of NFAT5 staining corresponds to the Ser 1197-phosphorylated form of nuclear factor of activated T cells 5 (NFAT5) (CY3, red). Von Willebrand staining (AlexaFluor488, green) identifies blood vessels. (**c, d**) Constitutive aldose reductase staining (AlexaFluor488, green) is observed in muscle fibers, which is selectively increased in the perifascicular atrophic muscle fibers. Double staining for the Heat Shock Protein 70 family (CY3, red) shows a very similar staining pattern. (**e**) Part of the perifascicular atrophic muscle fibers express CD56 (AlexaFluor488, green), presumably representing regenerating fibers. (**f**) Whereas muscle fibers of normal width are negative, part of the perifascicular atrophic muscle fibers are taurine transporter-positive (CY3, red). (**g**) Inducible nitric oxide synthase (CY3, red) is only present in the perifascicular atrophic fibers, and a subset of infiltrating CD68+ (AlexaFluor488, green) macrophages (arrow). (**h**) The chemokine CCL2 (AlexaFluor488, green) is detected in blood vessels (arrow) and in perifascicular atrophic muscle fibers. Scale bars = 50 μm.

CD206+ type 2 macrophages stained for SMIT. Scattered endomysial CD3+ T cells were either SMIT-positive or -negative, whereas CD3+ cells in larger infiltrates were mostly negative. The NFAT5 molecular target inducible nitric oxide synthase (iNOS) was localized to perifascicular atrophic muscle fibers, and to CD68+ tissue-infiltrating macrophages (Figure 1g). Another NFAT5 target, being chemokine CCL2, was localized to blood vessels, perivascular and endomysial inflammatory cells, and perifascicular atrophic muscle fibers (Figure 1h). No differences in staining patterns were observed between juvenile and adult DM.

Polymyositis/sporadic IBM

In PM and IBM also, strong myonuclear NFAT5 staining was observed in muscle fibers and part of the blood vessels. Most PM and IBM muscle fibers showed basal levels of AR, but were TauT-negative. Variable, often discontinuous, membranous SMIT staining was observed on muscle fibers (Figure 2h and j). Fibers with inclusions in the IBM muscle, identified as positive for the autophagic markers p62 and LC3B, contained SMIT aggregates (Figure 2j–o). Highly similar staining patterns were observed with both available anti-SMIT antibodies, and blocking studies with SMIT peptide showed the specificity of the staining inside the muscle fibers (Supplementary Figure S2). No staining for AR and TauT could be observed in the inclusions. The sarcoplasm of small fibers was more homogeneously SMIT-positive (Figure 2b), and strong staining was observed in satellite cells. The endothelial cell layer of arterioles and capillaries was mostly SMIT-positive. The vast majority of inflammatory cells were AR- and TauT-negative, whereas many CD68+ and CD206+ type 2 macrophages and CD3+ T cells were SMIT-positive (Figure 2b and c). Part of CD68+ macrophages invading non-necrotic muscle fibers were SMIT-positive, whereas invading CD8+ cytotoxic T cells were more often SMIT-positive (Figure 2h and i). The scattered and/or endomysial CD8+ T cells were mostly SMIT-negative.

Necrotizing autoimmune myopathy

Overall, the expression of osmolyte pathways was much lower in NAM muscle than in the other IIM. AR staining was strong in a subset of mostly small CD56+ muscle fibers; TauT was detected in a minority of CD56+ fibers and in these fibers, SMIT expression was even more infrequent (Figure 3). Inflammatory cells detected in NAM muscle were sparse, and these were largely AR, TauT and SMIT-negative.

Duchenne muscular dystrophy

In DMD tissues, myonuclear NFAT5 expression was most markedly increased, with strongest staining observed in small

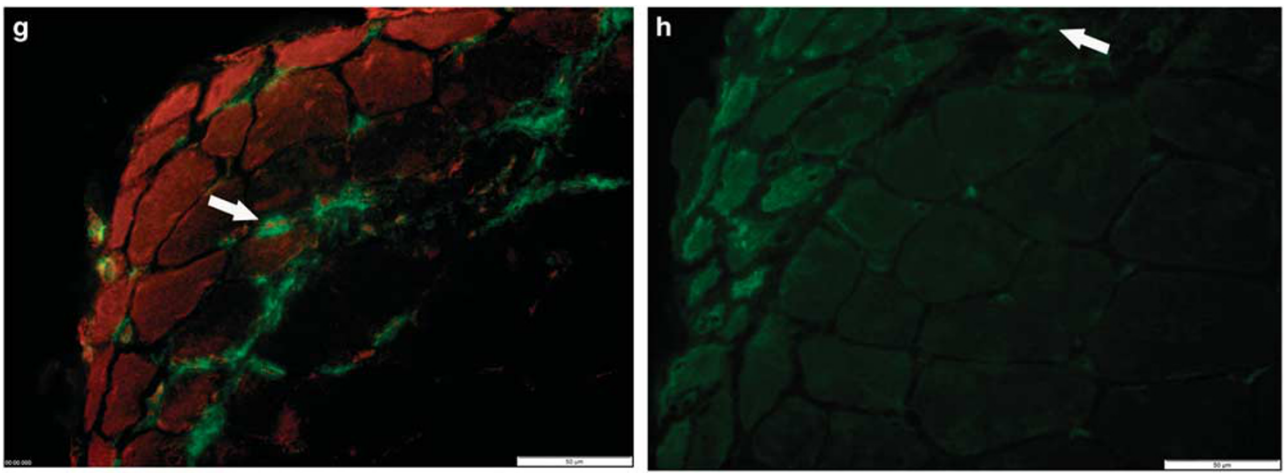
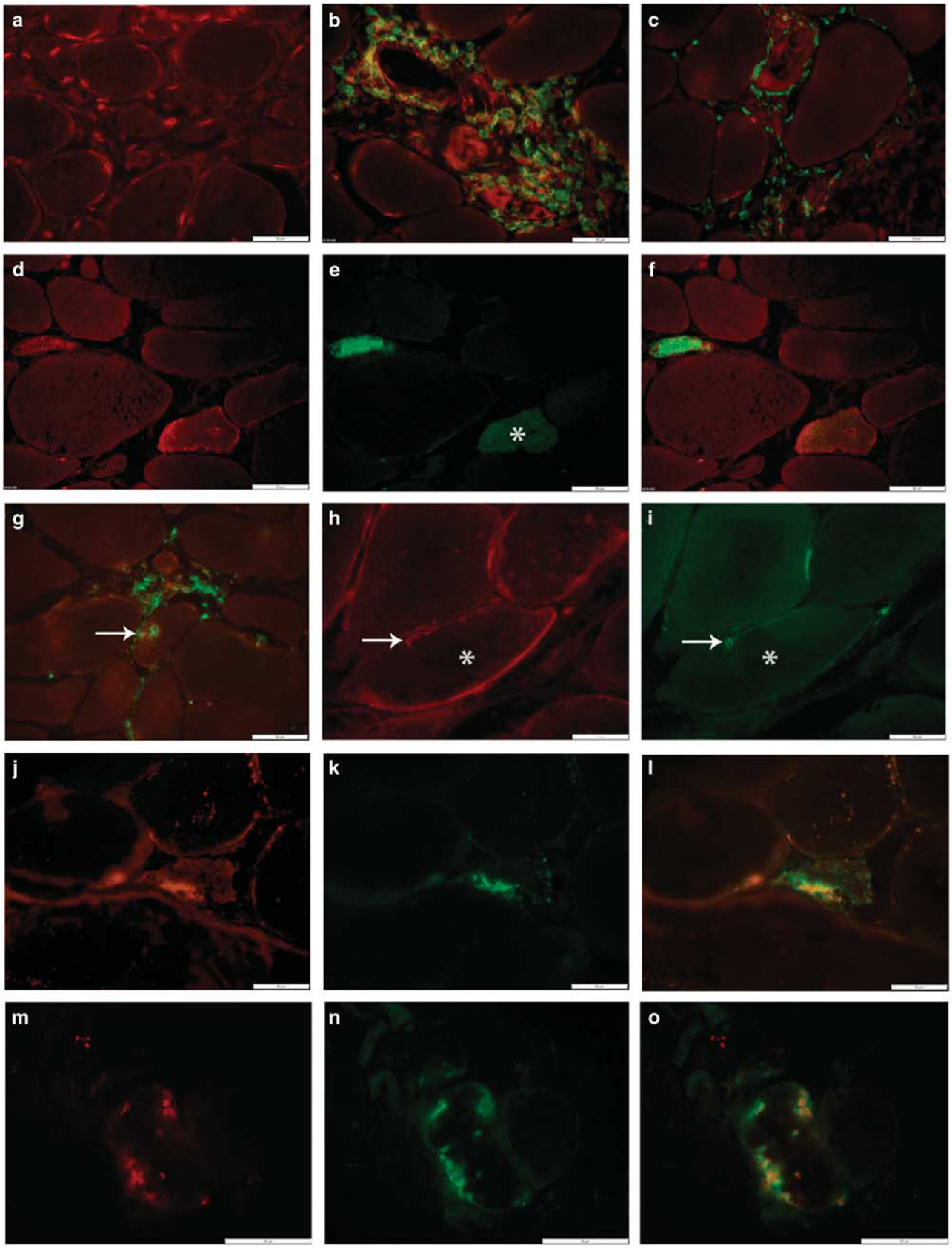


Figure 1 Continued.

Figure 2 The osmolyte pathway in polymyositis and sporadic inclusion body myositis. Polymyositis (**a**–**f**). (**a**) PM8: strong myonuclear staining for nuclear factor of activated T cells 5 (NFAT5; CY3, red) is accompanied by staining of the muscle surface. (**b**) PM2: sodium myo-inositol co-transporter (SMIT; CY3, red) shows discontinuous staining of the sarcolemma of muscle fibers with normal width, and strong sarcoplasmic staining in small fibers. The blood vessel endothelial layer also stains for SMIT, and part of perivascular CD3+ T cells are SMIT-positive. (**c**) PM3: rare CD206+ (AlexaFluor488, green) type 2 macrophages in and around a necrotic muscle fiber are SMIT (CY3, red)-positive. (**d**–**f**) PM3: aldose reductase staining (Cy3, red) is increased in the regenerating muscle fibers, identified as developmental MHC+ (AlexaFluor488, green), most pronounced in a late-stage regenerating fiber (asterisk). Sporadic inclusion body myositis (**g**–**o**). (**g**) IBM4: a subset of CD68+ (AlexaFluor488, green) macrophages in an endomysial infiltrate and a macrophage invading a non-necrotic muscle fiber (arrow) are SMIT (CY3, red)-positive. (**h**–**i**) IBM5: strong membranous SMIT (CY3, red) staining is present on a non-necrotic invaded muscle fiber (asterisk). A single invading CD8+ (AlexaFluor488, green) T cell (arrow) is SMIT-positive. (**j**–**l**) IBM7: SMIT (CY3, red) and p62 (AlexaFluor488, green) colocalize to muscle fiber inclusions. (**m**–**o**) IBM11: staining for SMIT (CY3, red) and LC3B (AlexaFluor488, green) localizes to muscle fibers containing inclusions. Scale bars = 50 μm.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fibers and central nuclei (Figure 4a). In addition, the sarcoplasm of a subset of small fibers stained for Ser 1197-phosphorylated NFAT5 (Figure 4b). This staining pattern mimicked staining for NFκB subunit p65 phosphorylated on Ser 536 (Figure 4c). Both HSP70 (Figure 4a) and AR (Figure 4d) upregulations were observed in a subset of muscle fibers of normal width, whereas in CD56+ small fibers mostly low expression could be observed (Figure 4d). In contrast, strong TauT expression was present in the CD56+ regenerating fibers, showing perinuclear accentuation (Figure 4e). Histochemical staining confirmed localization of TauT protein mostly to small fibers (Figure 4f). SMIT staining was detected in the sarcoplasm of CD56+ small fibers, whereas myonuclear and partial membranous staining was observed in many fibers. Inflammatory cells were AR- and TauT-negative, whereas the majority of CD68+ and a subset of CD206+ type 2 macrophages were SMIT-positive (Figure 4g and h). A minority of T cells were also SMIT-positive.

Quantitative Protein Data

In muscle tissue extracts and in extracts from primary myotubes and rhabdomyosarcoma cells cultured *in vitro*, NFAT5 was largely nuclear, whereas AR displayed mostly cytoplasmic expression (Supplementary Figure S3). In DMD myotubes, AR levels were high and could not be increased further by high-salt conditions (Supplementary Figure S4). Western blotting for AR, TauT, and SMIT in a selection of patients (Figure 5) showed that relative AR protein levels were 0.56 ± 0.10 ($n = 8$) in healthy muscle. Statistical analysis revealed that these levels were unchanged in a set of adult DM samples (0.45 ± 0.01, $n = 4$, $P > 0.05$), yet significantly increased in DMD (0.82 ± 0.06, $n = 2$, $P = 0.04$) and PM/IBM (0.89 ± 0.11, $n = 3$, $P = 0.01$) muscle protein samples. Whereas TauT and SMIT protein levels were below the detection limit in the normal muscle samples, low levels could be detected in the majority of patient samples. Western blotting for phosphorylated NFκB subunit p65 and p38 is shown in Figure 6. Relative phosphorylated p65 levels were significantly higher in IIM patients (0.63 ± 0.11, $n = 6$, $P = 0.0007$) compared with normal muscle samples (0.31 ± 0.08, $n = 4$). Phosphorylated p38 levels were also significantly higher in IIM patients (1.02 ± 0.40, $n = 6$, $P = 0.02$) than in normal muscle (0.52 ± 0.24, $n = 4$).

DISCUSSION

The ubiquitous transcription factor NFAT5 is constitutively expressed in skeletal muscle tissue[13] and in cultured myoblasts and myotubes.[14] Of the three NFAT5 targets involved in osmolyte accumulation we tested for this study, we found only AR to be present in substantial amounts in normal muscle, which confirms an earlier report[14] and points to a physiological role in muscle homeostasis.[15] Levels of TauT (The Human Protein Atlas: http://www.proteinatlas.org) and SMIT mRNA in the human skeletal muscle are low[16,17] and we could not detect the corresponding protein in normal muscle samples. However, TauT knockout mice suffer from muscle fiber atrophy and necrosis and reduced exercise endurance,[18,19] which implies that these factors could still be crucial factors for muscle recovery after damage. It has been observed that NFAT5 levels increase in the regenerating fibers of mouse tibial muscle following muscle tissue injury.[14] Our current data point to a general activation of the osmolyte pathway in regenerating muscle fibers in myositis.

Osmolyte Pathway Activation in DMD

In the mdx mouse model, decreased osmotic stability has long been recognized both in cultured myotubes and in isolated mature muscle fibers.[20] Resting cytosolic Ca^{2+} levels are increased and the impaired calcium homeostasis is further aggravated by exercise.[21] In addition, persistent (1.5-fold) sodium overload, and resulting muscle edema, has been shown in DMD patients.[22] Although an involvement in muscular dystrophy has long been suggested,[23] the precise role of osmolyte pathways in the disease remains poorly known. The general upregulation of AR in CD56− DMD muscle fibers we describe here fits with the assumption that in patients the muscle fibers are struggling to maintain physiological osmolarities because of leaky membranes. Our *in vitro* data, showing that the high AR protein levels in DMD myotubes could not be increased further by high-salt conditions, could also corroborate this assumption. A proteomic study of the heart muscle from 20-month-old mdx mice has also shown a modest yet significant 1.4-fold increase of AR protein levels.[24] In contrast to the increased expression we report here, significantly lower TauT levels have been found in mdx muscle (reviewed in De Luca *et al.*[25]), and taurine supplements were shown to ameliorate exercise-induced loss of muscle strength in these mice.[26] This discrepancy could reflect the unique features of human disease *vs* the murine disease model. In mdx mice, a limited window of enhanced muscle degeneration/regeneration occurs at an early age, whereas in DMD patients these processes persist throughout life.

Osmolyte Pathway Activation in DM

We report prominent expression of AR, TauT, and SMIT in the perifascicular atrophic fibers of DM, accompanied by increased/induced expression of other known NFAT5 molecular targets HSP70,[27] iNOS,[28] and CCL2.[29] As was the case earlier for iNOS,[30] we could, however, not confirm a significant increase of AR protein levels in DM muscle extracts via western blotting, presumably because of its expression being limited to the affected perifascicular areas.[31] The induction of CCL2, specifically in part of the perifascicular atrophic muscle fibers of DM, further corroborates earlier observations.[32] Presumably, CCL2 expression at perifascicular areas guides circulating monocytes to these areas, allowing characteristic local accumulation of responsive macrophages and T cells. The increase in HSP70 chaperone expression in the

perifascicular atrophic muscle fibers was particularly striking. Expression of these inflammation-associated factors represents both protective and catabolic tissue responses. Evidence exists that HSP induction precedes the accumulation of organic osmolytes, offering a way to protect the cells as the organic osmolytes accumulate. Later, HSP70 induction is

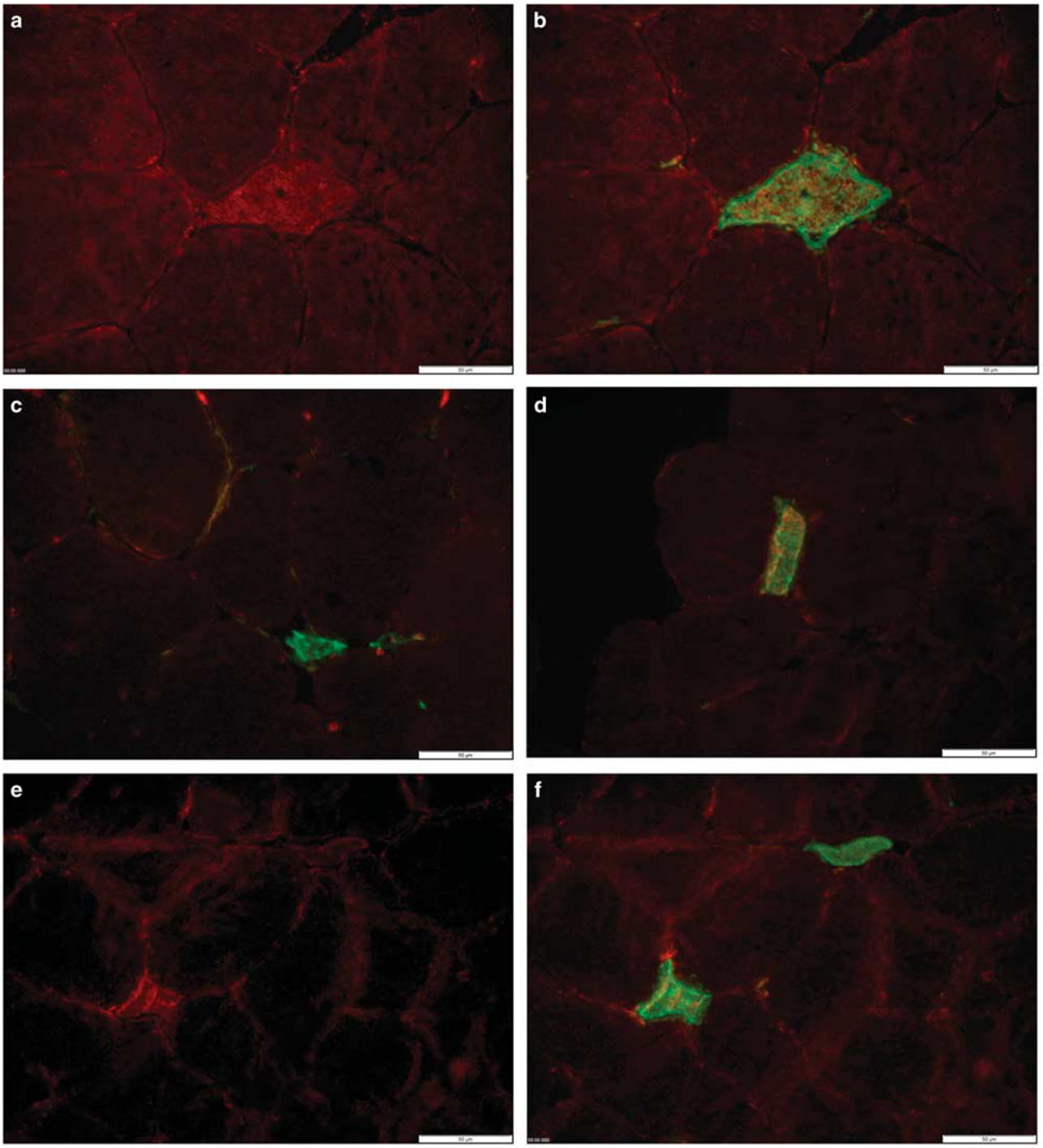

Figure 3 The osmolyte pathway in necrotizing autoimmune myopathy. (**a, b**) Necrotizing autoimmune myopathy (NAM) 2: immunostaining for aldose reductase (CY3, red) is increased in the CD56+ (AlexaFluor488, green) muscle fibers. (**c**) NAM3: a small CD56+ (AlexaFluor488, green) muscle fiber is taurine transporter (CY3, red)-negative. (**d**) NAM1: a small CD56+ (AlexaFluor488, green) muscle fiber is taurine transporter (CY3, red)-positive. (**e, f**) NAM1: of the two CD56+ (AlexaFluor44, green) muscle fibers in this microscopic field, only one is sodium myo-inositol co-transporter-positive (Cy3, red). Scale bars = 50 μm.

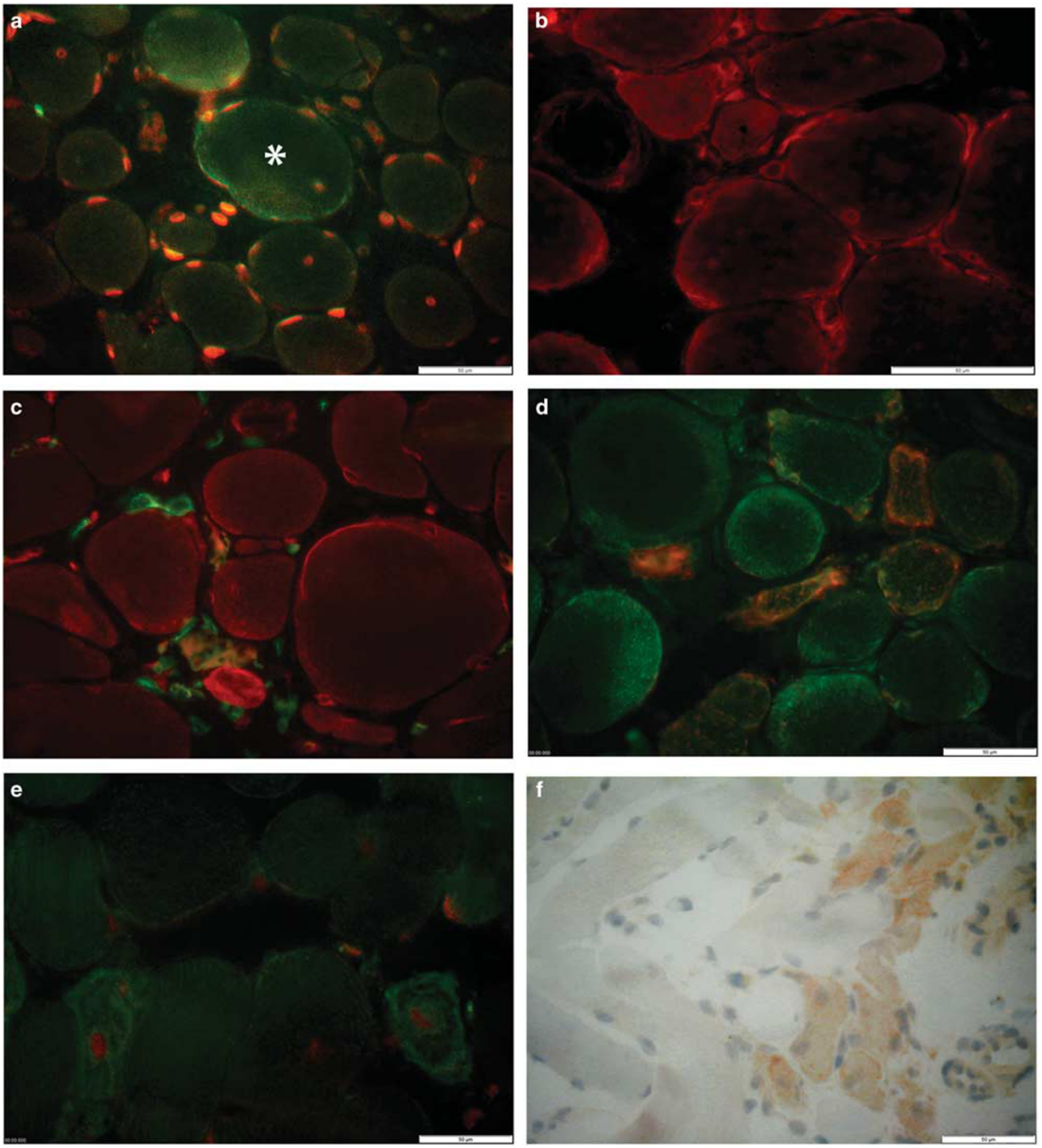

Figure 4 The osmolyte pathway and associated factors in Duchenne muscular dystrophy. (**a**) DMD5: strong myonuclear nuclear factor of activated T cells 5 (NFAT5; CY3, red) staining is present throughout the tissue, and is strongest in small fibers. Strong staining for HSP70 (AlexaFluor488, green) is mostly observed in muscle fibers with normal width and a hypercontracted fiber (asterisk). (**b**) DMD3: staining with a phospho-specific antibody shows myonuclear, membranous, and uneven sarcoplasmic staining corresponding to the Ser 1197-phosphorylated form of NFAT5 (CY3, red). (**c**) DMD2: strong sarcoplasmic staining for the nuclear factor κB (NFκB)-phosphorylated p65 subunit (CY3, red) is observed in small fibers. Discontinuous staining on muscle fiber membranes and staining on myonuclear membranes is also present. Part of CD206+ (AlexaFluor488, green) type 2 macrophages are p65-negative. (**d**) DMD6: strong aldose reductase staining (AlexaFluor488, green) is observed in many muscle fibers, but levels are not increased in the CD56+ (CY3, red) small fibers. (**e**, **f**) DMD4: TauT (CY3, red in **e**) is selectively expressed in the CD56+ (AlexaFluor488, green in **e**) fibers. (**f**) The expression of TauT in small fibers is confirmed with immunohistochemical staining for TauT (DAB, brown). (**g**) DMD5: SMIT (CY3, red) stains on the muscle fiber membrane and a subset of scattered CD68+ (AlexaFluor488, green) macrophages. (**h**) DMD1: in this section, lower-intensity staining for SMIT (CY3, red) is observed with more discrete membrane staining. The majority of CD206+ (AlexaFluor488, green) type 2 macrophages are SMIT-positive. Scale bars = 50 μm.

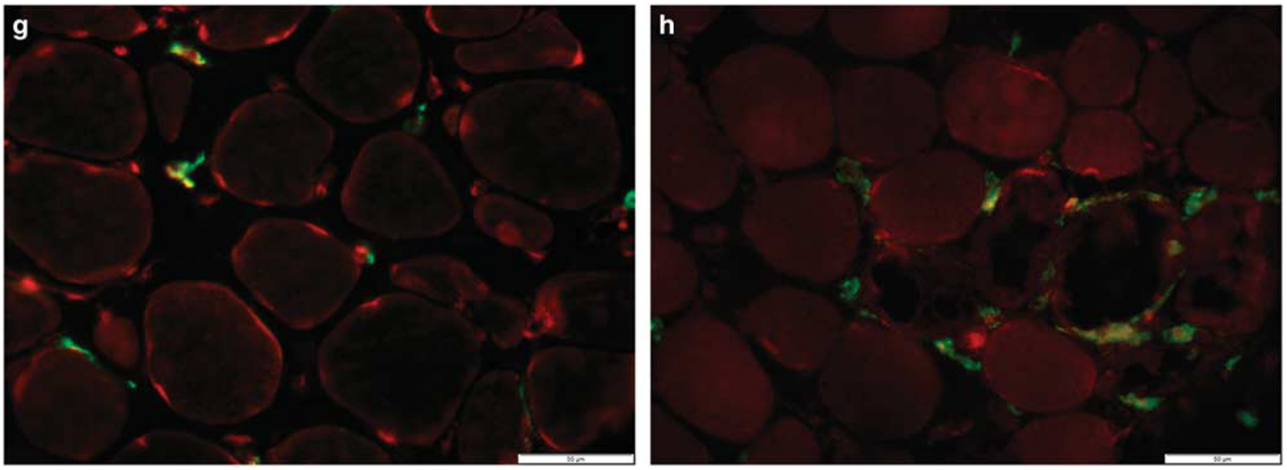

Figure 4 Continued.

mitigated and it returns to normal levels.[33] The question remains whether the activation of osmolyte pathways is beneficiary or, on the contrary, detrimental to these atrophic fibers. The protective effect of taurine supplementation against muscle damage suggests a positive effect where TauT is concerned.[34]

Osmolyte Pathway Activation in NAM, PM, and IBM

We observed AR upregulation and TauT and SMIT induction in regenerating muscle fibers of all IIM. In addition, SMIT was detected in the inclusions inside IBM muscle fibers. In IBM, unfolded/misfolded proteins form multiprotein aggregates inside muscle fibers likely through failing of autophagic clearance. The inclusions contain a multitude of proteins, among which are phosphorylated tau and amyloid-β,[35] resembling the composition of Alzheimer's disease plaques in the brain. The shuttle protein Sequestosome 1 (p62), which transports polyubiquitinated proteins toward the lysosomes for degradation, is an established immunohistochemical marker for IBM inclusions. When autophagic processes are disrupted, p62 accumulates with polyubiquitinated proteins through its ubiquitin-binding domain. In the aggregates inside IBM fibers, p62 colocalizes with phosphorylated tau.[36] We here report strong SMIT staining in muscle fiber inclusions colocalizing with p62 as well as with standard autophagic marker LC3B immunoreactivity. Although this could be due to nonselective trapping of the protein, myo-inositol metabolism has been linked to neurodegeneration before. Twofold increased levels have been observed in the brain of pre-dementia phase Alzheimer's disease.[37] A possible role for SMIT in degenerative processes is currently under exploration. The constitutive SMIT levels in the brain[17] were found moderately increased in the hippocampus of aged mice;[38] however, severity of tissue damage was not related to SMIT expression in TgCRND8, a mouse model of Alzheimer's disease-like amyloid pathology.[39]

SMIT is Expressed in Muscle-Infiltrating Immune Cells

We detected SMIT, but never AR or TauT, in part of the muscle-infiltrating macrophages and subsets of T cells of IIM and DMD, which points to a possible role for SMIT in inflammatory cell function. The relationship between NFAT5 and immune cell function has been put forward, and in macrophages, myo-inositol is believed to have a central role in cell volume regulation during phagocytosis. Infiltrating immune cells in murine muscle subjected to experimental muscle injury express NFAT5,[14] and SMIT protein levels have been shown to increase in liver macrophages subjected to hypertonic conditions.[40]

NFAT5 and NFκB Pathways are Possibly Linked in Myositis

NFκB activation has been established in muscular dystrophy, with mdx myotubes displaying increased NFκB activity and p65 translocation to the myonuclei, leading to iNOS expression. This process is thought to originate from increased intracellular resting calcium levels, and to involve p38 pathway activation.[41] We recently reported that both hyperosmotic conditions and pro-inflammatory cytokines were able to increase NFAT5 mRNA expression in DMD myotubes *in vitro*,[42] linking NFAT5 and NFκB pathways in dystrophic muscle cells. Besides its specific role in the hypertonic stress response, NFAT5 seems to also act as a more all-round stress factor functioning in close relationship with NFκB. The NFAT5 DNA complex shares many structural features with both the NFAT and NFκB families of transcription factors. Whereas the NFAT5 C-terminal interface is remarkably similar to that of NFκB, the N-terminal interface closely resembles that of NFAT1.[43] It is therefore likely that the two families regulate gene expression through shared enhancer elements, in which cytokines could act as important go-betweens. NFAT5 is a known inducer of LTβ expression, a cytokine that is expressed in IIM and DMD muscle tissue.[4] It has been established that LTβ signaling leads

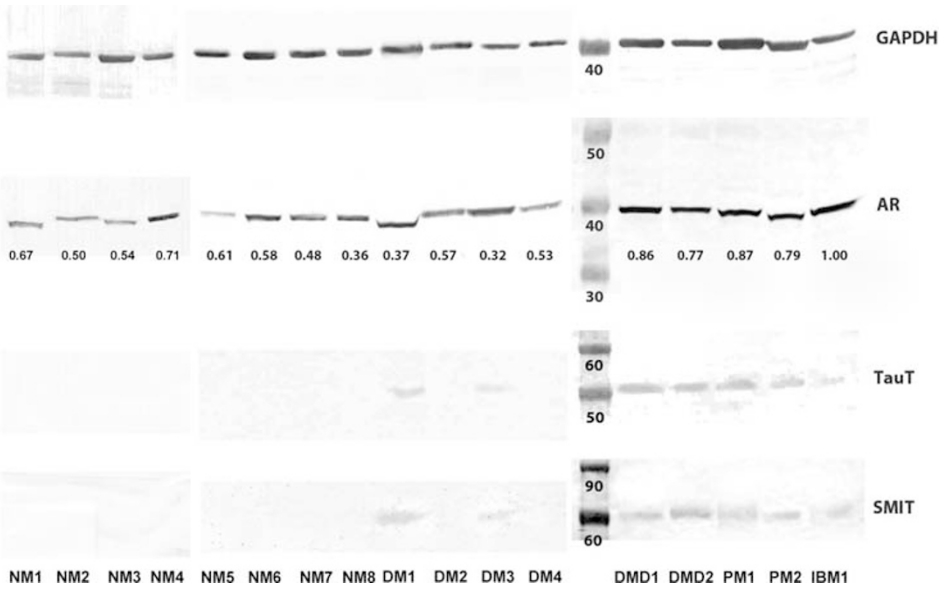

Figure 5 Western blotting for osmolyte pathways in skeletal muscle extracts. Protein bands for aldose reductase (AR), taurine transporter (TauT), and sodium myo-inositol co-transporter (SMIT) in eight normal control muscles (NM), four dermatomyositis (DM), two Duchenne muscular dystrophy (DMD), two polymyositis (PM), and one sporadic inclusion body myositis (IBM) muscle samples are shown. For AR, mean ± s.d. protein levels relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content are indicated.

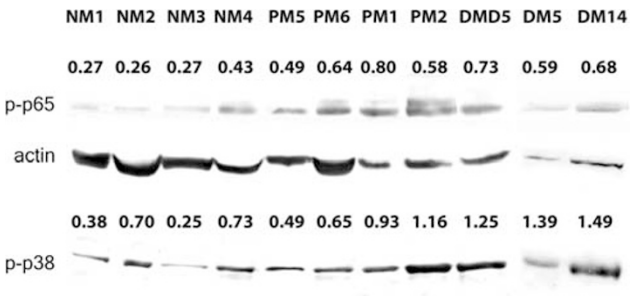

Figure 6 Western blotting detecting the phosphorylated forms of nuclear factor κB (NF*k*B) p65 subunit and MAP kinase p38 in skeletal muscle extracts. In four normal control muscle (NM), four polymyositis (PM), one Duchenne muscular dystrophy (DMD), and two dermatomyositis (DM) muscle samples, protein bands and the corresponding mean ± s.d. protein levels relative to actin content are indicated for phosphorylated p65 (p-p65) and phosphorylated p38 (p-p38).

to phosphorylation of NFκB on Ser 536 inside the transactivation domain 1 of p65, which is a crucial event for NFκB activation.[44] We observed striking accumulation of phosphorylated NFAT5 in perifascicular atrophic muscle fibers very similar to the staining pattern observed for the phosphorylated NFκB subunit p65.

The hypothesis has arisen of a complex relationship between cellular responses to either cytokines or osmotic stress, that find each other at the crossroads of NFAT5 and NFκB signaling. We report here that protein levels of phosphorylated p65 and p38 are both increased twofold in IIM and DMD. Hypertonicity-induced NFκB activity has been shown, which is dependent on Akt, p38, and NFAT5 activity. TNFα-induced NFκB activation on the other hand was hardly affected by NFAT5 silencing[45] and NFAT5 neutralization could not influence the T cell cytokine profile.[46] In this respect, the osmotic response element (ORE) or TonE sequences in gene promoters could represent the most important link between the two transcription factor pathways. ORE sequences have been reported in the promoter regions of the *AR*[47] and *SMIT* gene.[48] As ORE sequences have been identified in many other genes, including chaperones (HSP70 genes *HSPA4* and *HSPA5*), cytokines (IFNγ, IL-1α/β, IL-2, IL-6, IL-8, and LTβ; GeneCards: http://www.genecards.org), and chemokines.[29] NFκB proteins p50 and p65 can successfully bind the ORE sequence of the *AR* promoter, and the ORE complexes achieved through either TNFα or NaCl exposure are extremely similar, suggesting common mechanisms.

In conclusion, we here provide detailed immunolocalization, showing peculiar and differential expression patterns of osmolyte pathway members AR, TauT, and SMIT in IIM and DMD. Complex interactions between NFAT5 and NFκB pathways could be an important underlying regulatory mechanism. The activation of osmolyte pathways in muscle inflammation is relevant to disease management and may offer future therapeutic targets. Inhibition of AR, for instance, has been shown to prevent inflammation build-up in the allergic lung.[49]

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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