Int J Clin Exp Pathol 2016;9(4):4226-4233
www.ijcep.com /ISSN:1936-2625/IJCEP0021323

Original Article

Expression of adenosine receptors in muscle tissues from patients with idiopathic inflammatory myopathies

Xiao Yu[1], Fuchen Liu[2], Honghao Li[2], Chuanzhu Yan[2]

Departments of [1]Rheumatology, [2]Neurology and Neuromuscular Center, Qilu Hospital, Shandong University, Jinan 250012, P. R. China

Received December 7, 2015; Accepted February 17, 2016; Epub April 1, 2016; Published April 15, 2016

Abstract: This study aimed to determine the expression pattern of the adenosine receptor (AR) subtypes A_{2A}, A_{2B}, and A_3 in human idiopathic inflammatory myopathies (IIMs). A total of 27 patients diagnosed with IIMs were evaluated in this retrospective study, including 14 dermatomyositis, 9 polymyositis, and 4 inclusion body myositis cases. Fourteen control subjects without clinical and histological neuromuscular disorders were included. All patients and control subjects underwent muscle biopsy for diagnostic purposes. A_{2A}AR, A_{2B}AR, and A_3AR levels in skeletal muscle specimens were assessed by Western blotting and immunohistochemistry, and data were analyzed by independent sample *t* test. In human skeletal muscle tissues, A_{2A}AR, A_{2B}AR, and A_3AR were detected on the sarcolemma in the cytosol of type I fibers and in the cytosol of type II fibers, respectively. A_3AR was also detected in vascular smooth muscle cells. Fluorescence intensities of A_{2A}AR, A_{2B}AR, and A_3AR were higher in IIM patients than in controls. In agreement, more intense protein bands of A_{2A}AR and A_{2B}AR were obtained for IIM patients than for controls. However, no significant difference was observed in A_3AR bands between IIM patients and controls. CK levels and glucocorticoid treatment did not affect the expression of AR subtypes. In conclusion, expressions of A_{2A}AR, A_{2B}AR, and A_3AR in skeletal muscles were elevated in IIM patients but not affected by CK level or glucocorticoid treatment.

Keywords: Adenosine, adenosine receptor, idiopathic inflammatory myopathy, muscle biopsy

Introduction

Idiopathic inflammatory myopathies (IIMs) are a group of disorders of unknown etiology characterized by noninfectious inflammation of skeletal muscles [1]. The most prevalent IIM subtypes are polymyositis (PM), dermatomyositis (DM, typical skin rash), and inclusion body myositis (IBM, inclusion bodies within the nucleus or cytoplasm of muscle fibers) [2]. The main pathological feature of IIMs is intramuscular mononuclear cell infiltration and subsequent muscle fiber necrosis.

Adenosine is a signaling molecule in autocrine and paracrine pathways, and plays an important role in maintaining internal environment stability. Extracellular adenosine levels are enhanced both in patients with sepsis and in animal models with Crohn's disease [3-5]. Besides inhibiting oxidative burst and downregulating the expression of tumor necrosis factor-α and other inflammatory cytokines, adenosine is also a regulator of vascular tone [6] and plays an important role in regulating immune and inflammatory responses. Sattin and Rall [7] found that adenosine exerts its regulatory function only through binding to specific receptors on the cell surface.

Adenosine receptors (ARs) are G protein-coupled receptors with seven transmembrane domains. According to their molecular structures, ARs can be divided into four subtypes: A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR. Of these, A_1AR, A_{2A}AR, and A_{2B}AR have highly conserved sequences, with 80%-95% homology, while A_3AR has significant sequence variation [8]. Despite the highly similar molecular structures of ARs, the affinity to adenosine and mechanisms of action are different among various subtypes. At low concentrations, adenosine binds to A_1AR, A_{2A}AR, and A_3AR, while at higher concentrations, it favors selective binding with A_{2B}AR [9]. ARs can activate Gi and/or Gs proteins and modulate signal transduction path-

ways that change the concentrations of cAMP, Ca^{2+}, and other signaling molecules within the intercellular space [10].

Several studies have shown that A_3AR plays an important role in regulating the inflammatory response and reducing tissue damage [11, 12]. In addition, applying A_3AR agonists reduced serum creatine kinase (CK) levels [9]. However, Sajjadi et al. found that A_3AR is barely expressed in human skeletal muscle tissues [13]. Using immunohistochemistry and Western blotting methods, Lynge and Hellsten demonstrated that A_{2A}AR and A_{2B}AR were expressed both on the membrane and in the cytoplasm of skeletal muscle fibers, while A_1AR was not detected [14].

Despite this impressive wealth of knowledge, whether ARs are involved in IIMs remains unclear. Therefore, this study aimed to assess the roles of AR subtypes A_{2A}, A_{2B}, and A_3 in human IIMs.

A_{2A}AR, A_{2B}AR, and A_3AR were detected in muscle tissues from patients with IIMs, indicating AR implication in the immunopathogenesis of inflammatory myopathies. These findings provide a new direction for treating IIMs.

Materials and methods

Patients

Specimens were obtained from patients with clinical evidence of muscle diseases, and extracted from the biceps, quadriceps femoris, or deltoid muscles by biopsy. All specimens were collected at the Qilu Hospital, Shandong University, Jinan, P. R. China, between March 2009 and December 2011. The tissue collection protocol was approved by the Institutional Review Board of Qilu Hospital, and followed the recommended guidelines for the use of human subjects. All patients provided informed consent.

A total of 27 IIM patients, aged 48.1 ± 15.1 (range from 18 to 70) years, were included in this study. Among them, there were 14, 9, and 4 cases of DM, PM, and IBM, respectively. PM and DM diagnosis were consistent with the Bohan and Peter diagnostic criteria [15, 16]; IBM diagnosis conformed to the Griggs diagnostic criteria [17]. All patients were defined by symmetric proximal muscle weakness, elevated serum skeletal muscle enzymes before therapy, and muscle biopsy abnormality of interstitial mononuclear infiltrate. Twelve control subjects, aged 45.2 ± 14.5 (range from 22 to 68) years, were included from outpatient and hospitalized patients with muscle weakness clinically, and muscle biopsies were made for diagnosis. Control subjects were confirmed with no neuromuscular disorders clinically, and showed normal muscle tissues in histology.

All patients and controls were informed on the nature and possible risks of the experimental procedures before written informed consent was obtained. All procedures were performed according to the Declaration of Helsinki, and the study was approved by the Medical Ethics Committee of Qilu Hospital, Shandong University, Jinan, China.

Immunofluorescence and immunohistochemistry

Specimens were cooled in isopentane and snap-frozen in liquid nitrogen, sectioned, and processed with routine hematoxylin-eosin and adenosine triphosphatase staining protocols.

Immunofluorescence and histochemical staining was conducted as described by Sebastian et al. in their study [18]. Immunolocalization of A_{2A}AR, A_{2B}AR, and A_3AR was visualized by indirect detection via rhodamine (TRITC) signals under a fluorescence microscope. Frozen tissue specimens were sectioned at 5-μm thickness. Nonspecific binding was blocked with 1% normal goat serum albumin, and tissue sections were probed with rabbit anti-human A_{2A}AR (10 μg/mL, Millipore, Darmstadt, Germany), A_{2B}AR (10 μg/mL, Millipore), and A_3AR (10 μg/mL, Bioworld, USA). In negative controls, the primary antibody was replaced with preimmune rabbit immunoglobulin G (IgG) at the same concentration. Rhodamine (TRITC)-conjugated Affinipure goat anti-rabbit IgG secondary antibodies (15 μg/mL, Zhongshan Golden Bridge, Beijing, China) were used.

The immunofluorescence- and immunohistochemistry-positive signals (using integrated optic density, IOD) were analyzed with the NIH ImageJ 1.49 V imaging analysis software (http://imagej.nih.gov/ij/).

Table 1. Clinical and pathological characteristics of patients and control subjects

	Patients	Control	*P* value
Age (year)	48.1 ± 15.1	45.2 ± 14.5	$P > 0.05$
Men (*n*, %)	11, 40.7	5, 41.6	$P > 0.05$
IIMs			
DM (*n*, %)	14, 51.9	–	–
PM (*n*, %)	9, 33.3	–	–
IBM (*n*, %)	4, 14.8	–	–
Course			
0~12 months (*n*, %)	15, 55.6	–	–
12~24 months (*n*, %)	5, 18.5	–	–
More than 24 months (*n*, %)	7, 25.9	–	–
Normal CK level (*n*, %)	4, 14.8	12, 100	$P < 0.001$
Hormonal therapy (*n*, %)	17, 63.0	–	–
Biopsy parts			
Left biceps brachii (*n*, %)	23, 85.2	10, 83.3	$P > 0.05$
Left deltoid (*n*, %)	3, 11.1	1, 8.3	$P > 0.05$
Left quadriceps femoris (*n*, %)	1, 3.7	1, 8.3	$P > 0.05$
Necrotic fibers			
Generous (*n*, %)	4, 14.8	–	–
Common (*n*, %)	6, 22.2	–	–
Consperse (*n*, %)	9, 33.3	–	–
Occasional (*n*, %)	3, 11.1	–	–
No (*n*, %)	5, 18.5	12, 100	$P < 0.001$
Focal inflammatory cell infiltration (n, %)	23, 85.2	–	–
Perifascicular atrophy (*n*, %)	11, 40.7	–	–

CK, Creatine kinase; DM, Dermatomyositis; IBM, Inclusion body myositis; IIMs, Idiopathic inflammatory myopathies; PM, Polymyositis.

Western blotting

Western blot analysis was conducted as described in a previous study [18]. Briefly, skeletal muscle tissues were homogenized and lysed by sonication in the following buffer: 0.5 M Tris-HCl, 10% glycerol, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 0.05% bromophenol blue. After centrifugation, protein concentrations of the supernatants were determined using the bovine serum albumin (Fraction V; Zhongshan Golden Bridge) as standard. Protein samples (30 μg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrically transferred onto polyvinylidene difluoride membranes. The membranes were consecutively probed with anti A_{2A}AR, A_{2B}AR, and A_3AR primary antibodies (dilution, 1:1000; Millipore), and then with horse radish peroxidase-conjugated secondary antibodies (dilution, 1:5000; Zhongshan Golden Bridge). Antiβ-actin (dilution, 1:200; Sigma-Aldrich, MO, USA) antibodies served as a loading control. Proteins were visualized using enhanced chemiluminescence reagents (Millipore). Immunoreactive signals were analyzed by densitometry using the NIH ImageJ 1.49 V imaging analysis software.

Statistical analyses

Statistical analyses were performed using the SPSS17.0 statistical software (IBM, NY, USA). Measurement data were expressed by mean ± standard deviation and analyzed using the analysis of variance, followed by independent sample *t* test. Category data were shown as (*n*, %) and analyzed with chi-square analysis. $P < 0.05$ was considered statistically significant.

Results

Clinical and pathological data of the patients

The clinical and pathological characteristics of the 27 IIM patients and 12 control subjects are summarized in **Table 1**. The course of disease varied from 20 days to 8 years, and CK levels were 42-6829 U/L. The CK level was normal in 4 IIM patients and 12 control subjects. Of the patients, 17 were administered glucocorticoids equivalent to ≥ 1 mg/kg/day of dehydrocortisone for more than a month. There were 23 biceps brachii, 3 deltoid, and 1 musculus quadriceps fexoris cases. Histopathology identified 23 cases with focal inflammatory cell infiltration and 22 cases with necrotic muscle fibers; 11 of the 14 DM patients had perifascicular atrophy.

AR immunofluorescence detection

A_{2A}AR signals were detected on the sarcolemma of muscle fibers (**Figure 1A** and **1B**). Moreover, A_{2B}AR signals were found in the cyto-

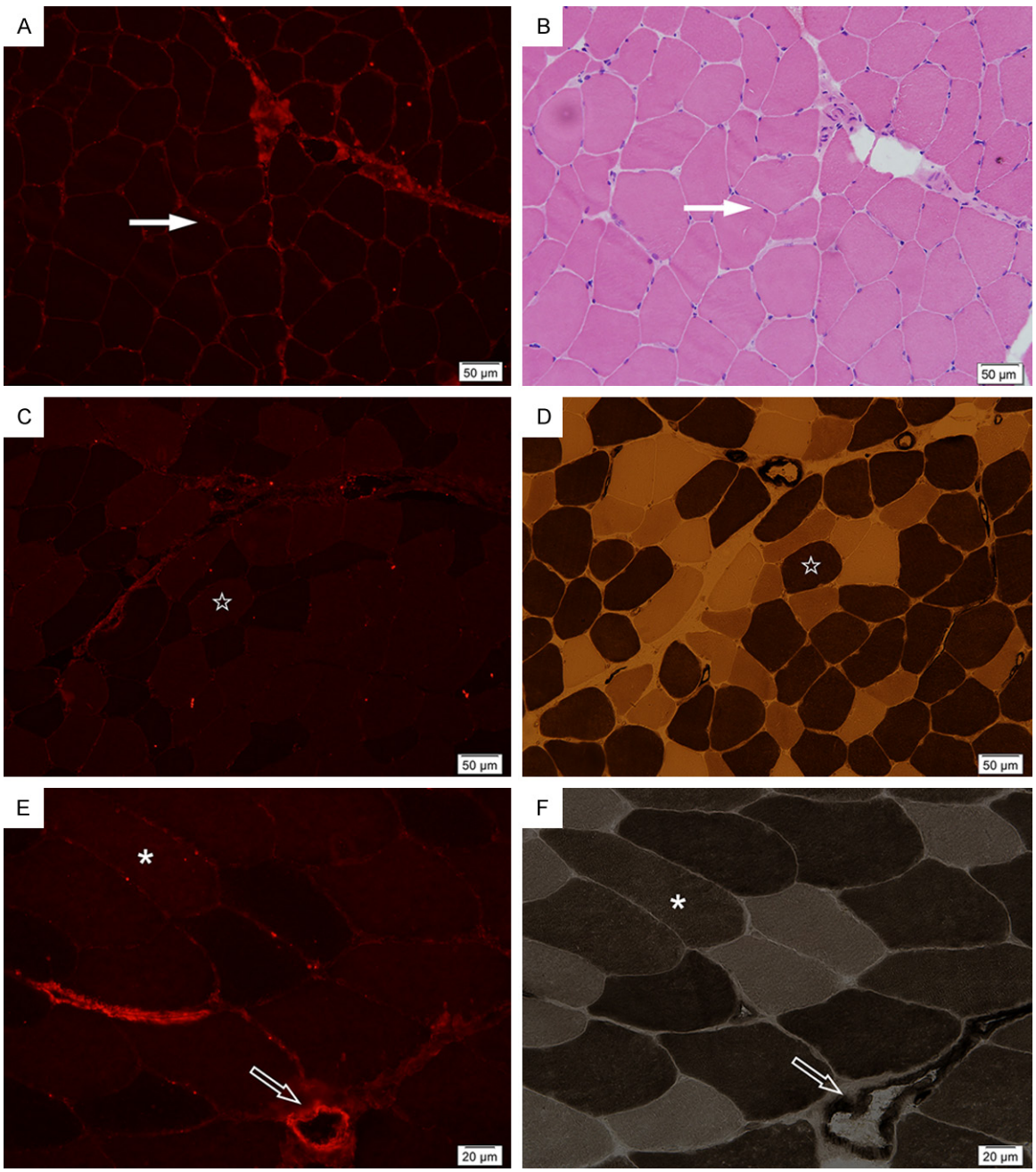

Figure 1. Localization of $A_{2A}AR$, $A_{2B}AR$, and A_3AR in human skeletal muscle tissues. $A_{2A}AR$ signals were found on the sarcolemma with a fluorescence intensity of + (A); muscle tissues stained with HE (B). $A_{2B}AR$ signals were observed in the cytosol of type I fibers with fluorescence intensity of +, corresponding to dark type I fibers stained with ATPase (pH 4.7) (C, D). A_3AR signals were localized in the cytosol of type II fibers, corresponding to dark type II fibers stained with ATPase (pH 10.4); fluorescence intensity in the cytosol was + and that in the vascular wall +++ (E, F). The bold arrows (➔) represent the sarcolemma, stars (☆) represent cytosol of type I muscle fibers, asterisks (*) represent cytosol of type II fibers, and hollow arrows (⇒) represent the vascular wall. ATPase, adenosine triphosphatase; HE, hematoxylin-eosin.

sol of type I fibers and appeared to be absent in type II fibers (**Figure 1C** and **1D**). The A_3AR signals were obtained in the cytosol of type II fibers and vascular smooth muscle cells, but the protein was absent in type I fibers (**Figure 1E** and **1F**).

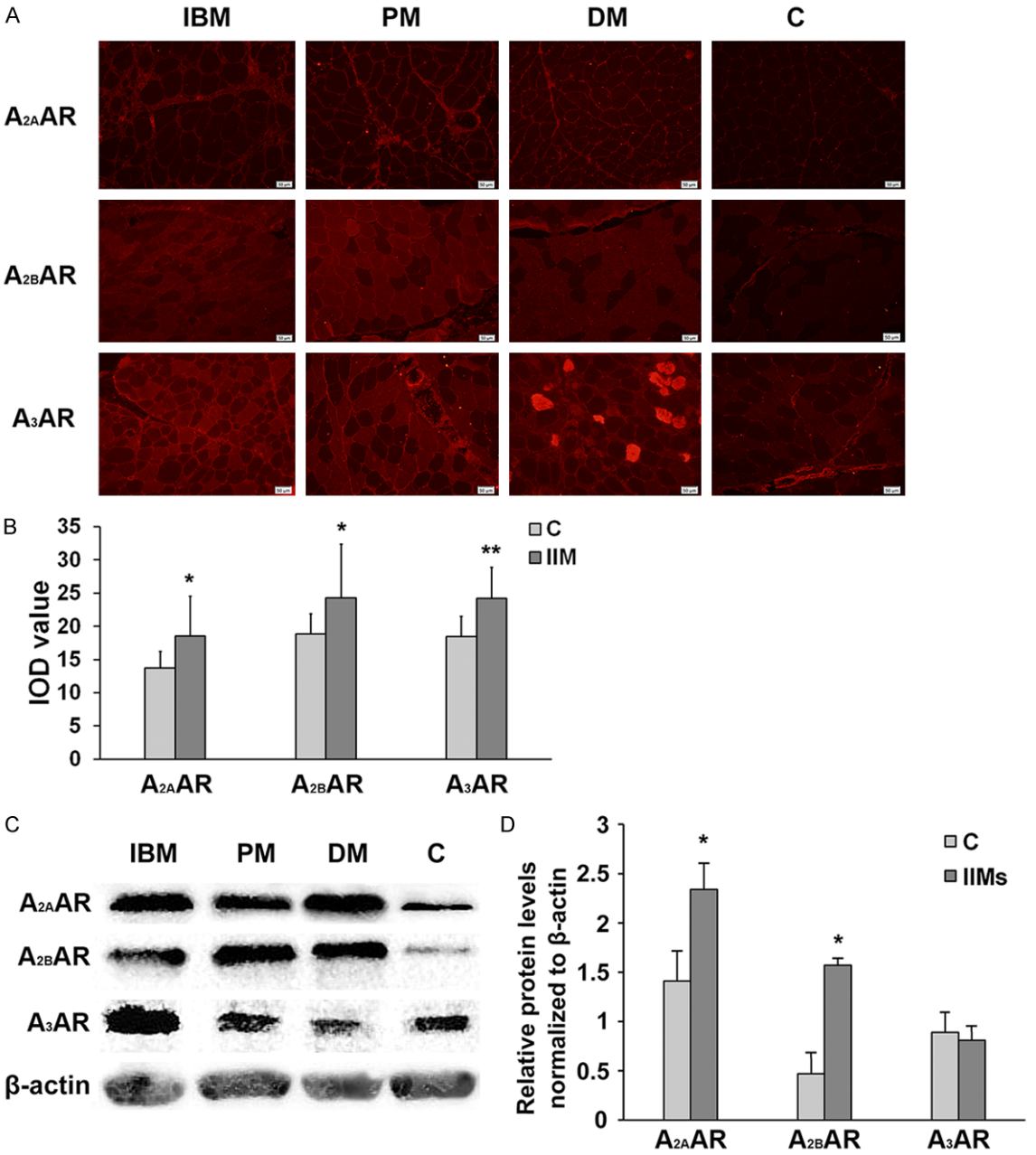

Figure 2. $A_{2A}AR$, $A_{2B}AR$, and A_3AR levels in IIM patients and controls. Fluorescence signals of $A_{2A}AR$, $A_{2B}AR$, and A_3AR in frozen skeletal muscle sections from IBM, PM, DM, and control subjects (A). IOD values of fluorescence signals of $A_{2A}AR$, $A_{2B}AR$, and A_3AR in frozen skeletal muscle sections from IIM patients and control subjects (B). Protein bands of $A_{2A}AR$, A_2BAR, and A_3AR in IBM, PM, DM, and control subjects (C). Semiquantitative Western blot analysis of $A_{2A}AR$ and $A_{2B}AR$ signals between IIM patients and controls (D). *$P < 0.05$ and **$P < 0.01$ vs controls. DM, dermatomyositis; IBM, inclusion body myositis; IIM, idiopathic inflammatory myopathy; IOD, integrated optic density; PM, polymyositis.

Interestingly, the $A_{2A}AR$, $A_{2B}AR$, and A_3AR subtypes were detected in human skeletal muscles, showing different immunofluorescence intensities between IIM patients and controls ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively) (**Figure 2A** and **2B**).

AR levels detected by Western blot

The relative intensities of $A_{2A}AR$, $A_{2B}AR$, and A_3AR protein bands in samples from IIM patients were compared with those obtained for the control group (**Figure 2C** and **2D**). The

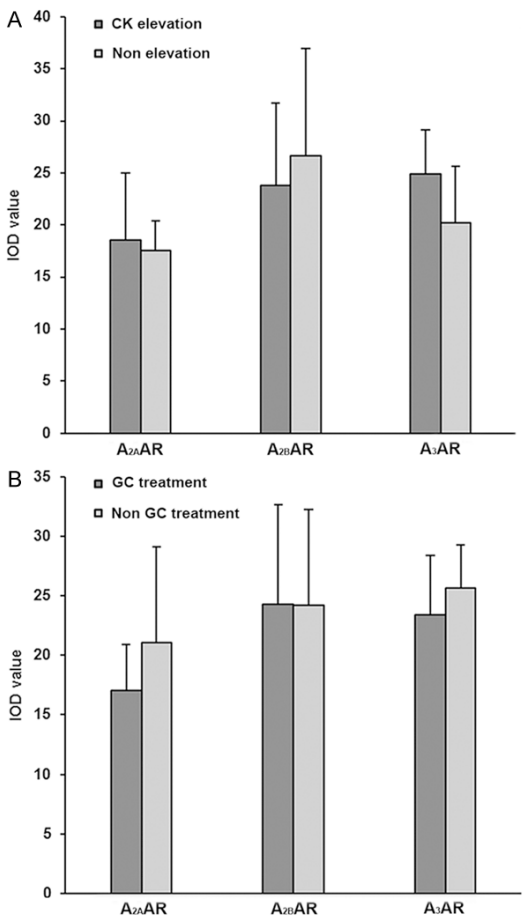

Figure 3. $A_{2A}AR$, $A_{2B}AR$, and A_3AR levels in IIM patients with and without CK level elevation and glucocorticoid treatment. $A_{2A}AR$, $A_{2B}AR$, and A_3AR levels in IIM patients with and without CK level elevation (A). $A_{2A}AR$, $A_{2B}AR$, and A_3AR levels in IIM patients with and without glucocorticoid treatment (B). CK, creatine kinase; GC, glucocorticoid; IIM, idiopathic inflammatory myopathy.

values for $A_{2A}AR$ and $A_{2B}AR$ protein bands were higher in IIM patients than in controls ($P < 0.01$), but A_3R showed no statistically significant difference ($P > 0.05$).

Effect of CK level elevation and glucocorticoid treatment

The differences in $A_{2A}AR$, $A_{2B}AR$, and A_3AR fluorescence intensities in nonnecrotic muscle fibers between the CK elevation and nonelevation groups were not statistically significant ($P > 0.05$) (**Figure 3A**). Also, no significant differences were obtained in $A_{2A}AR$, $A_{2B}AR$, and A_3AR fluorescence intensities in nonnecrotic muscle fibers between patients using glucocorticoids and those not using these drugs ($P > 0.05$) (**Figure 3B**).

Discussion

This study demonstrated that $A_{2A}AR$ signals were detected on the sarcolemma of muscle fibers, $A_{2B}AR$ signals in the cytosol of type I fibers, and A_3AR signals in the cytosol of type II fibers and vascular smooth muscle cells (**Figure 1**). The $A_{2A}AR$, $A_{2B}AR$, and A_3AR subtypes were detected in human skeletal muscles, showing different immunofluorescence intensities between IIM patients and controls (**Figure 2**). The study also revealed that more intense protein bands of $A_{2A}AR$ and $A_{2B}AR$ were obtained for IIM patients than for controls. No statistically significant differences were found in $A_{2A}AR$, $A_{2B}AR$, and A_3AR fluorescence intensities in nonnecrotic muscle fibers between the CK elevation and nonelevation groups and also between patients using glucocorticoids and those not using these drugs ($P > 0.05$) (**Figure 3**).

In 1993, Sajjadi et al. reported that A_3AR was only expressed in the human placenta, lung, heart, pancreas, and kidney, and not in skeletal muscle tissues [13]. In the current study, an A_3AR band of 38 kD was identified by Western blotting, and positive A_3AR immunofluorescence signals were detected in muscle fibers. Both methods yielded concordant data, confirming A_3AR expression in human skeletal muscle tissues.

A_3AR is thought to participate in repairing injured cells. Indeed, previous animal studies showed that $A_{2B}AR$ and A_3AR levels rose rapidly 3 days after cold injury, with more evident A_3AR expression [9]. In addition, applying selective A_3AR agonists reduces necrotic cell percentages after ischemia and reperfusion injury in mouse skeletal muscles [19]. Furthermore, A_3AR induction decreases blood CK levels in mice with abnormal movement [20]. Therefore, increased A_3AR expression is considered to be protective for the skeletal muscle. In the present study, nonnecrotic type II muscle fibers expressed higher A_3AR amounts in IIM patients than in controls as assessed by immunohistochemistry; however, no difference was found in A_3AR amounts between skeletal tissue specimens from IIM patients and those from controls according to Western blot analysis. Data in this study suggest that type II fibers express

high A_3AR levels for self-protection from injury in the state of nonspecific inflammatory response. However, type II to type I fiber ratios might be affected by disease status and drug use, among others.

As suggested by Lynge et al. [14], this study found that A_{2B}AR was found in the cytosol of type I muscle fibers and not of type II muscle fibers; moreover, this study detected A_{2A}AR on the sarcolemma of muscle fibers, in agreement with the report by Lynge et al.

A few limitations of this study should be mentioned. First of all, the sample size was limited in this single-center study. In addition, the AR activity in IIM patients was not assessed. Therefore, more in-depth and large multicenter studies are needed to confirm the findings of the present study.

In conclusion, the human skeletal muscle expresses A_{2A}AR, A_{2B}AR, and A_3AR. The AR levels increased in IIM patients than in control subjects. The CK levels or glucocorticoid treatment did not affect the expression of ARs. Further research should be carried out to verify if appropriate levels of adenosine and AR might be an effective method to alleviate IIM-induced injury of the skeletal muscle.

Acknowledgements

We appreciate the technical guidance provided by Dandan Zhao (Laboratory of Neuromuscular Disorders and Department of Neurology, Qilu Hospital, Shandong University, Jinan, China) in slicing and staining the specimens.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Chuanzhu Yan, Department of Neurology and Neuromuscular Center, Qilu Hospital, Shandong University, 107 West Wenhua Road, Jinan 250012, P. R. China. Tel: +86-18560081682; Fax: +86-531-82169217; E-mail: czyan726@sina.com

References

[1] Rayavarapu S, Coley W, Kinder TB and Nagaraju K. Idiopathic inflammatory myopathies: pathogenic mechanisms of muscle weakness. Skelet Muscle 2013; 3: 13-13.

[2] Dimachkie MM and Barohn RJ. Inclusion body myositis. Semin Neurol 2012; 32: 237-245.

[3] Antonioli L, Fornai M, Colucci R, Ghisu N, Blandizzi C and Del Tacca M. A2a receptors mediate inhibitory effects of adenosine on colonic motility in the presence of experimental colitis. Inflamm Bowel Dis 2006; 12: 117-122.

[4] Li ZW and Xun Q. Recent progress in relationship between adenosine, adenosine receptors and sepsis. Med Recapit 2008; 14: 3361-3364.

[5] Ribe D, Sawbridge D, Thakur S, Hussey M, Ledent C, Kitchen I, Hourani S and Li JM. Adenosine A2A receptor signaling regulation of cardiac NADPH oxidase activity. Free Radic Biol Med 2008; 44: 1433-1442.

[6] Borowiec A, Lechward K, Tkacz-Stachowska K and Skladanowski AC. Adenosine as a metabolic regulator of tissue function: production of adenosine by cytoplasmic 5'-nucleotidases. Acta Biochim Pol 2006; 53: 269-278.

[7] Sattin A and Rall TW. The effect of adenosine and adenine nucleotides on the cyclic adenosine 3', 5'-phosphate content of guinea pig cerebral cortex slices. Mol Pharmacol 1970; 6: 13-23.

[8] Hasko G, Pacher P, Deitch EA and Vizi ES. Shaping of monocyte and macrophage function by adenosine receptors. Pharmacol Ther 2007; 113: 264-275.

[9] Warren GL, Hulderman T, Liston A and Simeonova PP. Toll-like and adenosine receptor expression in injured skeletal muscle. Muscle Nerve 2011; 44: 85-92.

[10] Cordeaux Y, Ijzerman AP and Hill SJ. Coupling of the human A1 adenosine receptor to different heterotrimeric G proteins: evidence for agonist-specific G protein activation. Br J Pharmacol 2004; 143: 705-714.

[11] Thiel M, Caldwell CC and Sitkovsky MV. The critical role of adenosine A2A receptors in downregulation of inflammation and immunity in the pathogenesis of infectious diseases. Microbes Infect 2003; 5: 515-526.

[12] Sands WA and Palmer TM. Adenosine receptors and the control of endothelial cell function in inflammatory disease. Immunol Lett 2005; 101: 1-11.

[13] Sajjadi FG and Firestein GS. cDNA cloning and sequence analysis of the human A3 adenosine receptor. Biochim Biophys Acta 1993; 1179: 105-107.

[14] Lynge J and Hellsten Y. Distribution of adenosine A1, A2A and A2B receptors in human skeletal muscle. Acta Physiol Scand 2000; 169: 283-290.

[15] Bohan A and Peter JB. Polymyositis and dermatomyositis (second of two parts). N Engl J Med 1975; 292: 403-407.

[16] Bohan A and Peter JB. Polymyositis and dermatomyositis (first of two parts). N Engl J Med 1975; 292: 344-347.
[17] Griggs RC, Askanas V, DiMauro S, Engel A, Karpati G, Mendell JR and Rowland LP. Inclusion body myositis and myopathies. Ann Neurol 1995; 38: 705-713.
[18] Gehlert S, Bungartz G, Willkomm L, Korkmaz Y, Pfannkuche K, Schiffer T, Bloch W and Suhr F. Intense resistance exercise induces early and transient increases in ryanodine receptor 1 phosphorylation in human skeletal muscle. PLoS One 2012; 7: e49326.
[19] Zheng J, Wang R, Zambraski E, Wu D, Jacobson KA and Liang BT. Protective roles of adenosine A1, A2A, and A3 receptors in skeletal muscle ischemia and reperfusion injury. Am J Physiol Heart Circ Physiol 2007; 293: H3685-3691.
[20] Wang R, Urso ML, Zambraski EJ, Rader EP, Campbell KP and Liang BT. Adenosine A(3) receptor stimulation induces protection of skeletal muscle from eccentric exercise-mediated injury. Am J Physiol Regul Integr Comp Physiol 2010; 299: R259-267.