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1 Short Communications

2 **Increased dystrophin mRNA and protein levels in atrophic skeletal muscles in**
3 **streptozotocin-induced diabetic rat**

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18

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21

22 Running Title: Dystrophin and muscle atrophy in diabetes

23 **Abstract**

24 Severe diabetes frequently induces skeletal muscle atrophy, and dystrophin disruption has
25 been implicated in the pathogenesis of skeletal muscle atrophy. We hypothesized that the
26 downregulation of dystrophin expression causes diabetic-induced muscle atrophy, and
27 investigated whether dystrophin mRNA and protein levels are altered in the atrophic muscles
28 of diabetic rats. Rats received a single intravenous injection of streptozotocin (STZ) (45
29 mg/kg body weight). Slow-twitch soleus and fast-twitch extensor digitorum longus muscles
30 were dissected from each rat 4 or 12 weeks after the STZ injection. The STZ group had
31 significantly higher blood glucose levels and lower body weights than the control group. The
32 relative muscle weight per body weight was also lower in the STZ group than in the control
33 group, and these changes accompanied a reduction in glucose transporter 4. The
34 phosphorylation of Akt Ser⁴⁷³ and p70 S6 kinase Thr³⁸⁹ was lower in the soleus and extensor
35 digitorum longus muscles of the diabetic rats than in those of the control rats. In contrast,
36 dystrophin mRNA and protein expression were higher in the muscles of the diabetic rats than
37 in those of the control rats. A histochemical study showed that the localization of dystrophin
38 did not differ between the muscles of the control and diabetic rats. Our data suggest that the
39 downregulation of dystrophin is not a general characteristic associated with skeletal muscle in
40 diabetes.

41

42 **Keywords:** dystrophin, atrophic muscle, hyperglycemia, insulin, streptozotocin

43 **Abstract**

44 重度の糖尿病状態では進行性の骨格筋萎縮を発症することが報告されている。また骨格筋構
45 成蛋白質の 1 つであるジストロフィンの欠損は骨格筋萎縮を誘発する。本研究では糖尿病性
46 筋萎縮の進行にはジストロフィン欠損が関与しているという仮説を立て、筋萎縮を呈するス
47 トレプトゾトシン (STZ) 誘発性糖尿病モデル動物を用いて、萎縮骨格筋におけるジストロ
48 フィン mRNA およびタンパク発現量を測定した。8 週齢の雄性 Sprague-Dawley ラットに生理
49 食塩水に溶解した STZ (45 mg/kg body weight) を尾静脈投与した群を STZ 群とし、生理食塩
50 水を投与した群を対照群とした。投与 4 または 12 週間後にヒラメ筋および長指伸筋を摘出す
51 るとともに血液を採取した。STZ 群では対照群に対し、血糖値の上昇および体重減少、体重
52 あたりのヒラメ筋および長指伸筋重量の低下が認められた。また筋萎縮に伴って glucose
53 transporter 4 タンパク発現量の減少が認められた。STZ 群の萎縮骨格筋では対照群に比べ、Akt
54 Ser⁴⁷³リン酸化、p70 S6 kinase Thr³⁸⁹リン酸化が減少した。一方、ジストロフィン mRNA およ
55 びタンパク発現量がともに増加した。免疫組織化学的検討の結果から、ジストロフィンの局
56 在は両群に差はなかった。以上の結果から、糖尿病状態における骨格筋萎縮にジストロフィ
57 ン欠損は関与していないことが示唆される。

58

59 江川達郎、増田慎也、後藤勝正、林達也

60 **Introduction**

61 Dystrophin is a structural protein that connects the cytoskeleton of a muscle fiber to the
62 surrounding extracellular matrix through the cell membrane. Mutation of dystrophin disrupts
63 the mechanical linkage and signaling pathway, resulting in membrane damage, necrosis, and
64 eventual muscle atrophy ¹⁾. Dystrophin dysfunction is also implicated in muscle wasting of
65 cancer cachexia ^{2, 3)}, which is associated with impaired insulin action in skeletal muscle ⁴⁾.

66 Poorly controlled diabetes affects protein metabolism ⁵⁾ and induces skeletal muscle
67 atrophy ⁶⁾. Although several studies have shown that reduced protein synthesis contributes to
68 it ^{7, 8)}, the precise mechanism of diabetic-induced muscle atrophy is not understood. Using
69 streptozotocin (STZ)-induced diabetic rats, recent studies have demonstrated that reduced
70 Akt/ mammalian target of rapamycin (mTOR) signaling is associated with diabetic-induced
71 muscle atrophy ⁹⁾. Risson et al. ¹⁰⁾ showed that the inactivation of mTOR leads to advanced
72 myopathy, with a strong reduction in the muscle dystrophin content.

73 We hypothesized that the downregulation of dystrophin expression might be associated
74 with diabetic-induced muscle atrophy. Thus, the primary purpose of this study was to
75 examine the expression of dystrophin mRNA and protein in the atrophic muscles of
76 STZ-induced diabetic rats. We also examined the changes in dystrophin localization in these
77 rats by histochemical analysis.

78 **Materials and methods**

79 **Animals**

80 All protocols for animal use and euthanasia followed the Guiding Principles for the Care
81 and Use of Animals in the Field of Physiological Sciences (Physiological Society of Japan), in
82 accordance with international guidelines, and were approved by the Kyoto University
83 Graduate School of Human and Environmental Studies. Male Sprague Dawley rats (8 weeks
84 old) were housed in an animal room maintained at 22–24 °C on a 12:12-h light–dark cycle
85 and were fed a standard laboratory diet and water ad libitum.

86

87 **STZ treatment**

88 Diabetes was induced with STZ (Sigma, St Louis, MO, USA). In brief, the animals
89 received a single intravenous injection of STZ (45 mg/kg body weight) via the tail vein. The
90 control animals received an equivalent volume of saline. Blood glucose levels were measured
91 using the glucose oxidase method, with an automated blood glucose analyzer (GluTestAce,
92 Sanwa Chemical Research Institute Co., Inc., Nagoya, Japan). Diabetes was defined as a
93 nonfasting glucose level of ≥ 300 mg/dl.

94

95 **Muscle sampling**

96 The animals were anesthetized with pentobarbital sodium (50 mg/kg body weight) and
97 killed 4 or 12 weeks after the STZ or saline injection. The soleus (SOL) and extensor
98 digitorum longus (EDL) muscles were dissected from each rat, weighed, immediately frozen
99 in liquid nitrogen, and stored at –80 °C for immunoblotting and Real-time RT-PCR analysis.
100 Some muscles were frozen in 2-methylbutane cooled with solid CO₂ and stored at –80 °C for
101 histochemical analysis.

102

103 Immunoblotting analysis

104 Total protein from muscles were extracted as we described previously ^{11, 12)} and subjected
105 to immunoblotting analyses. Blots were incubated with rabbit-anti Akt Ser⁴⁷³ (9271, Cell
106 Signaling Technology, Danvers, MA), rabbit-anti Akt (9272, Cell Signaling), rabbit-anti p70
107 S6 kinase (p70S6K) Thr³⁸⁹ (9205, Cell Signaling Technology, Danvers, MA), rabbit-anti
108 p70S6K (9202, Cell Signaling), rabbit anti-glucose transporter (GLUT) 4 (AB1346;
109 Chemicon, Temecula, CA, USA), mouse anti-dystrophin antibody (MAB1692; Chemicon).
110 The signal was detected by using ECL reagents (GE Healthcare, Buckinghamshire, UK). The
111 intensity of the signals was quantified using MultiAnalyst software (Bio-Rad Laboratories,
112 Berkeley, CA, USA). Every blot was duplicated and the mean value was adopted for each
113 sample.

114

115 Real-time RT-PCR analysis

116 Real-time RT-PCR analysis was performed according to Yasuhara et al. ¹³⁾. Briefly, total
117 RNA was extracted from muscles using the miRNeasy Mini kit (Qiagen, Hiden, Germany).
118 Samples were reverse-transcribed using PrimeScript RT Master Mix (Perfect Real Time) for
119 mRNA (Takara Bio, Otsu, Japan). Synthesized cDNA was applied to real-time reverse
120 transcription-PCR (Thermal Cycler Dice Real Time System IIMRQ, Takara Bio) using Takara
121 SYBR Premix Ex Taq II for mRNA, and analyzed with Takara Thermal Cycler Dice Real
122 Time System Software Ver. 4.00. To normalize the amount of total RNA present in each
123 reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA for mRNA was used
124 as an internal standard. The primers were designed by using the Takara Bio Perfect Real Time
125 Support System (Takara Bio). Primers used for detection of rat cDNA were as follows:
126 Dystrophin, 5'-AGGCCAAGTGTAACATCTGTAAGGA-3' (forward) and
127 5'-CCTTAGCAACTCGGCCAGAA-3' (reverse); GAPDH,

128 5'-GGCACAGTCAAGGCTGAGAATG-3' (forward) and

129 5'-ATGGTGGTGAAGACGCCAGTA-3' (reverse).

130

131 Histochemical analysis

132 The histochemical analyses used to detect dystrophin were as we described previously ¹¹).

133 The sections were incubated with mouse anti-dystrophin antibody (MAB1692; Chemicon),

134 and stained with 3,3'-diaminobenzidine. Histochemical analysis was performed using a

135 microscope (Optiphot-2, Nikon, Tokyo, Japan) with a charge-coupled device camera and a

136 computerized image processing system (Image/J 1.36b). To calculate the mean cross-sectional

137 area of the muscle fibers, 100-150 fibers were randomly picked up from each of the muscles.

138

139 Statistical analysis

140 The results are presented as means \pm SE. Means were compared with two-way ANOVA,

141 followed by a post hoc comparison with Scheffé's test as appropriate. $P < 0.05$ was considered

142 statistically significant.

143 **Results**

144 Glucose levels, body weight, muscle weight, GLUT4 levels

145 The STZ group had significantly higher blood glucose levels and lower body weights
146 than the control group at 4 and 12 weeks (Table 1). The muscle weight of the STZ group was
147 significantly lower than that of the control group at 4 and 12 weeks. The relative muscle
148 weight per body weight was also lower in the STZ group than in the control group, except for
149 the SOL at 12 week (P=0.08).

150 It has been shown that GLUT4 expression is greatly reduced in some conditions leading
151 to muscle atrophy such as disuse¹⁴, aging¹⁵, denervation¹⁶, and diabetes¹⁷. In accordance
152 with these studies, the GLUT4 levels in SOL and EDL muscles were reduced in the STZ
153 group compared with the control group (Figure 1).

154

155 Akt and p70S6K phosphorylation

156 Akt Ser⁴⁷³ phosphorylation was significantly reduced in the SOL and EDL muscles of the
157 STZ group at 12 weeks (Figure 2A). The change at 4 weeks was not statistical significant
158 (Figure 2A). p70S6K is major substrate for mTOR, and the phosphorylation of p70S6K
159 Thr³⁸⁹ in the STZ group was clearly reduced compared with that in the control group at both
160 4 and 12 weeks (Figure 2B).

161

162 Dystrophin mRNA and protein levels

163 The dystrophin mRNA and protein levels were higher in the SOL of the STZ group than
164 in the control group at both 4 and 12 weeks, and the mRNA and protein levels were also
165 higher in the EDL of the STZ group than in the control group at 12 weeks (Figure 3A and 3B).

166 A histochemical analysis showed marked fiber atrophy in the 12 weeks diabetic group
167 compared with the control group (SOL, control = $4807 \pm 275 \mu\text{m}^2$ vs. STZ = 2060 ± 106 ;

168 EDL, control = 3305 ± 187 vs. STZ = 1144 ± 38 , $p < 0.01$, respectively; $n = 4$ per group), but
169 dystrophin was expressed at the sarcolemma of all muscle fibers in the SOL and EDL muscles
170 of the STZ and control groups at 12 weeks (Figure 3C).

171 **Discussion**

172 The STZ-induced diabetic animal model has often been used to study muscle atrophy in
173 diabetes ^{9, 18, 19}). We have shown for the first time that the dystrophin mRNA and protein
174 levels are increased in the atrophic muscles of STZ-induced diabetic rats (Figure 3A), which
175 are characterized by insulin-deficient hyperglycemia, low body weight, and low muscle
176 weight (Table 1). Low muscle weight per body weight (Table 1) and a reduction in GLUT4
177 levels (Figure 1) indicate that skeletal muscle atrophy was induced in our diabetic rats,
178 because low GLUT4 expression is seen in some atrophic muscles including STZ-induced
179 diabetes ¹⁴⁻¹⁷). Contrary to our findings, Mulvey et al. ²⁰) reported that the expression of
180 dystrophin is reduced in the skeletal muscles of type 2 diabetic Goto-Kakizaki rats. They
181 speculated that the loss of dystrophin is at least partly responsible for the abnormal
182 characteristics of the skeletal muscle in diabetes. However, their diabetic rats did not exhibit
183 structural abnormalities, such as muscle atrophy.

184 Although the exact function of dystrophin is not completely understood, it is known to
185 play an important role in regulating the structural stability of the muscle cell membrane ^{1, 21}).
186 The signal transduction cascade from dystrophin has also been implicated in cell survival and
187 defense mechanisms, and in the regulation of the balance between cell survival and cell death
188 ^{21, 22}). There is also a functional link between dystrophin and cell growth signaling ¹⁰). It is
189 noteworthy that dystrophin is elevated at the sarcolemma of cardiac myocytes in failing
190 myocardium, and this is interpreted as a mechanism to preserve cellular stability and integrity
191 ²³). Therefore, the increased expression of dystrophin might be a compensatory response that
192 defend the muscle structure and functions against muscle atrophy under severely diabetic
193 conditions. Further study is required to clarify the role of dystrophin upregulation in
194 STZ-induced diabetes.

195 Consistent with a previous study that demonstrated that Akt/mTOR signaling is

196 associated with diabetic-induced muscle atrophy ⁹⁾, we confirmed that Akt Ser⁴⁷³ and p70S6K
197 Thr³⁸⁹ phosphorylation are reduced in atrophic muscles during diabetes (Figure 2). It has been
198 demonstrated that the inactivation of mTOR leads to advanced myopathy, with a strong
199 reduction in the muscle dystrophin content ¹⁰⁾. However, we found that dystrophin levels were
200 upregulated in the atrophic muscles of diabetic rats (Figure 3) although Akt/mTOR/p70S6K
201 signaling was downregulated (Figure 2). This implies that the downregulation of mTOR
202 signaling does not always cause a reduction in the dystrophin content of muscles.

203 Taken together, our data suggest that a reduced dystrophin content is not a common
204 feature of atrophic skeletal muscle, and that the pathogenesis of diabetic-induced muscle
205 atrophy cannot be attributed to the downregulation of dystrophin levels. Other mechanisms
206 such as the disturbance of protein synthesis signaling ⁹⁾ and the increased expression of genes
207 involved in protein degradation ²⁴⁾ may be crucial modulators of diabetic muscle atrophy.

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Table 1. Body weight, plasma glucose concentration, muscle weight in control and STZ-induced diabetic rats.

Period (week)	Control			STZ		
	0	4	12	0	4	12
Body weight (g)	191 ± 4	334 ± 8	531 ± 18	190 ± 4	280 ± 4 †	276 ± 4 †
Plasma glucose (mg/dL)	56 ± 2	76 ± 5	76 ± 3	70 ± 6	476 ± 22 †	490 ± 22 †
SOL muscle weight (mg)	—	140 ± 5	221 ± 4	—	100 ± 4 †	107 ± 5 †
weight/body weight	—	0.42 ± 0.011	0.42 ± 0.016	—	0.36 ± 0.010 *	0.38 ± 0.012
EDL muscle weight (mg)	—	154 ± 4	217 ± 6	—	94 ± 10 †	87 ± 5 †
weight/body weight	—	0.46 ± 0.016	0.40 ± 0.016	—	0.33 ± 0.024 †	0.32 ± 0.012 †
number of animals	14	4	10	12	3	9

282 Values are mean ± SE.

283 * P<0.05, † P<0.01 Significantly different from corresponding control group.

284 **Figure legend**

285 **Figure 1**

286 GLUT4 levels in SOL and EDL muscle. The muscle isolated from control and STZ groups
287 was subjected to immunoblot analysis. Fold increases are expressed relative to the level of
288 signal in the muscle of normal group (4 weeks). Equal protein loading and transfer was
289 confirmed by Coomassie brilliant blue (CBB) staining of a 200-kDa protein. Representative
290 immunoblots are shown. Values are mean \pm SE. *P<0.05, †P<0.01 vs. corresponding control
291 group.

292

293 **Figure 2**

294 Akt Ser⁴⁷³ phosphorylation (A), p70S6K Thr³⁸⁹ phosphorylation (B) levels in SOL and EDL
295 muscle. The muscle isolated from control and STZ groups was subjected to immunoblot
296 analysis. Fold increases are expressed relative to the level of signal in the muscle of normal
297 group (4 weeks). Representative immunoblots are shown. Values are mean \pm SE. *P<0.05,
298 †P<0.01 vs. corresponding control group.

299

300 **Figure 3**

301 Dystrophin mRNA (A) and protein (B) levels in SOL and EDL muscle. The muscle isolated
302 from control and STZ groups was subjected to real-time RT-PCR and immunoblot analysis.
303 Fold increases are expressed relative to the levels in the muscle of normal group (4 weeks).
304 Equal protein loading and transfer for immunoblot analysis was confirmed by CBB staining
305 of a 200-kDa protein. Representative immunoblots are shown. Values are mean \pm SE. *P<0.05,
306 †P<0.01 vs. corresponding control group. Histochemical analysis of dystrophin localization
307 (C). Representative transverse sections of SOL and EDL muscle dissected from 12-week
308 experimental groups are shown. Scale bars indicate 25 μ m.

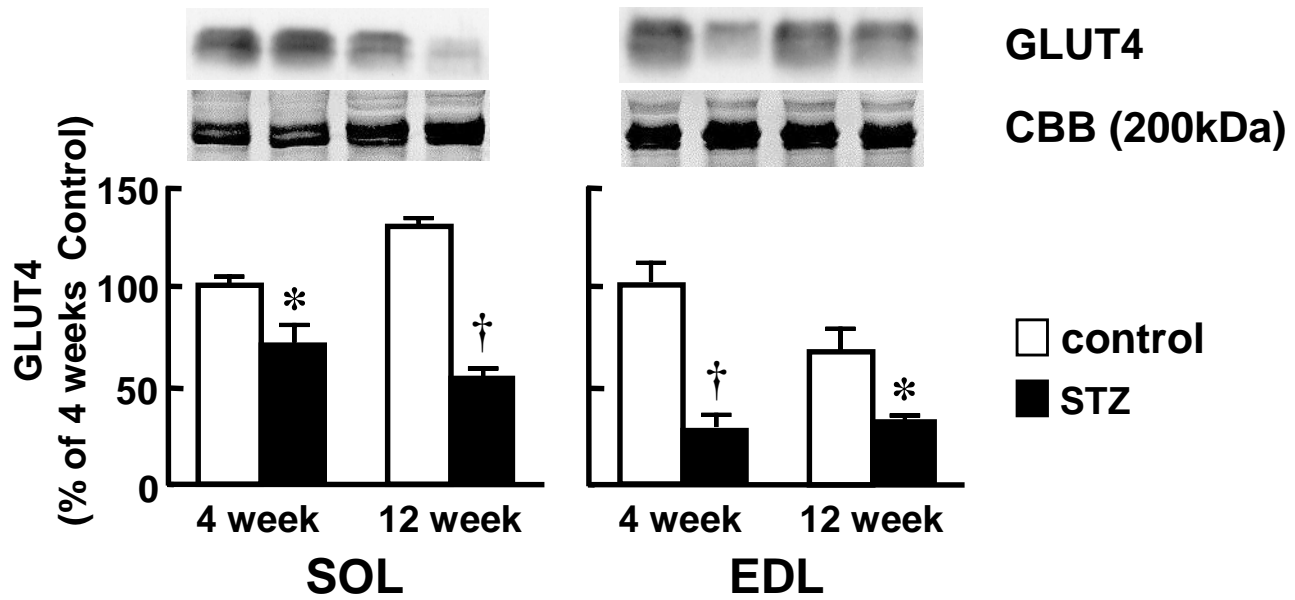


Figure 1

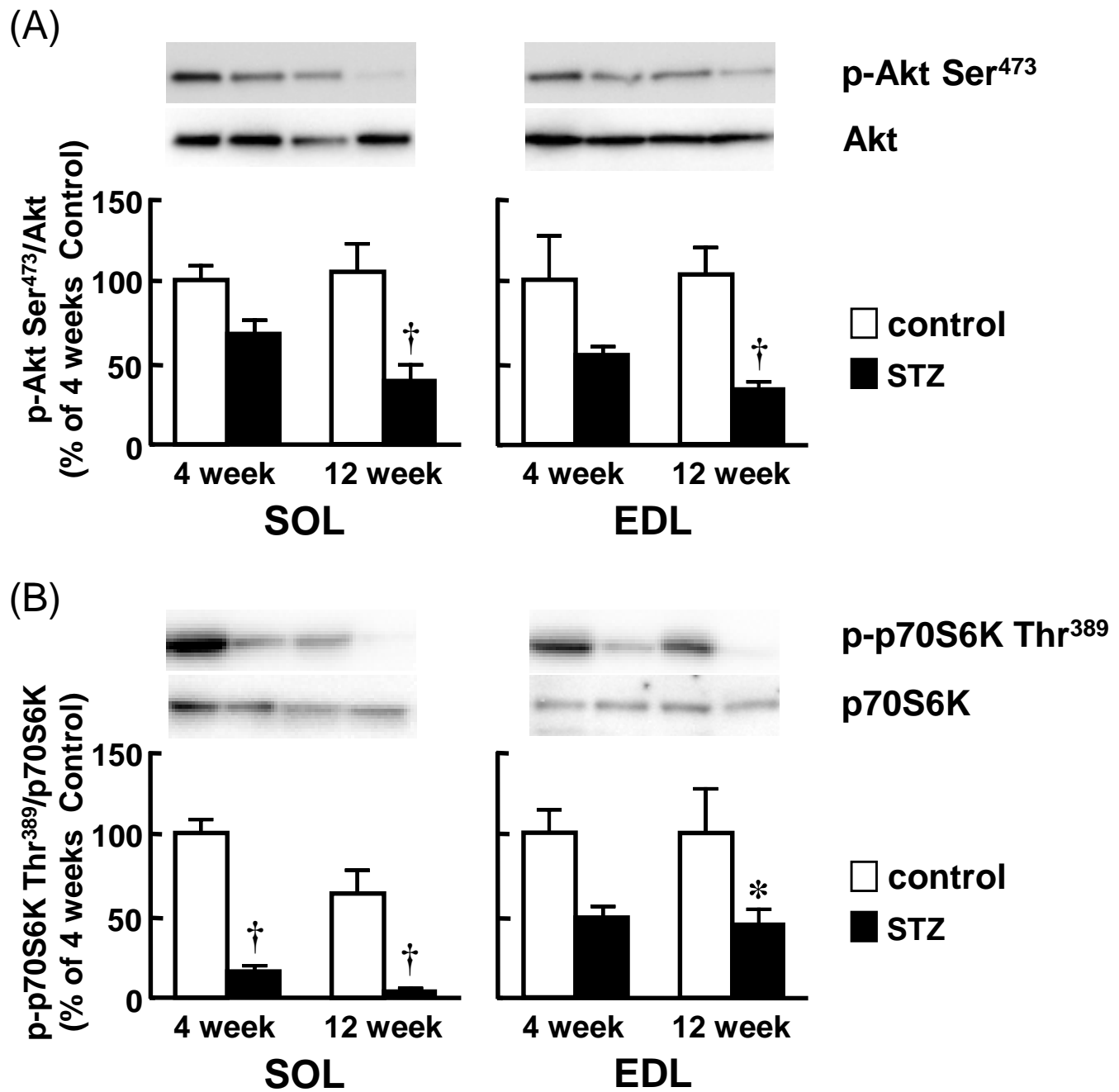


Figure 2

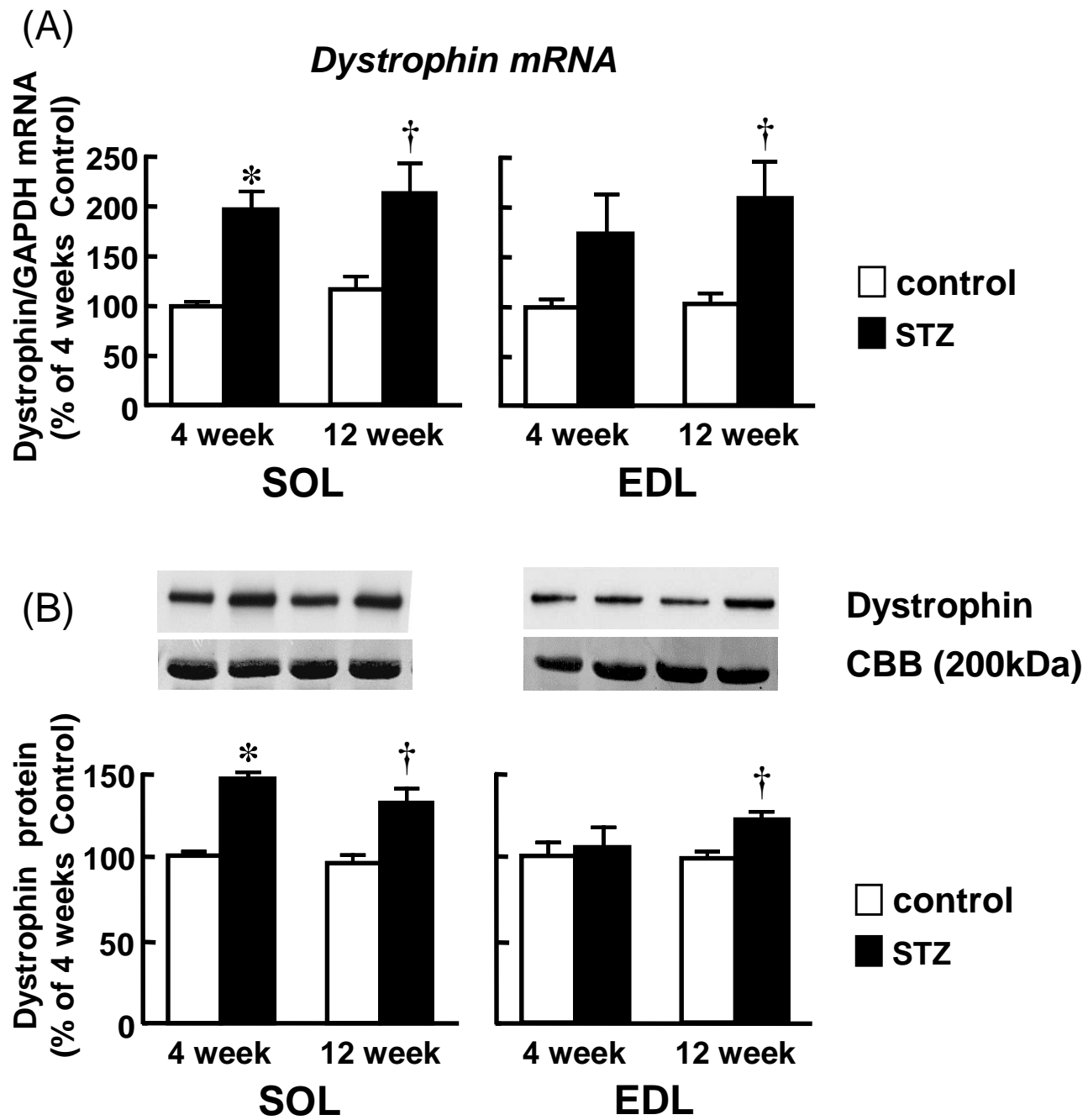


Figure 3

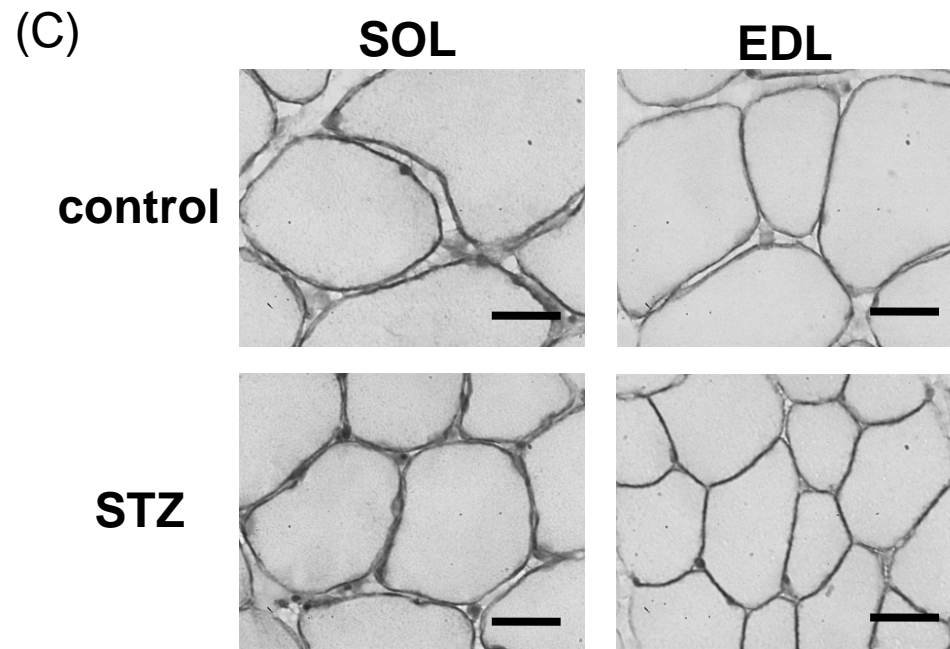


Figure 3