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*Short communication*

# Streptozotocin-induced diabetes alters the oligomerization pattern of acetylcholinesterase in rat skeletal muscle

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**Abstract**

*Aims/hypothesis.* Diabetes mellitus has a serious effect on most of the properties of skeletal muscles. Changes in neuromuscular transmission are also involved in propagating the disease.
*Methods.* In our experiments, acetylcholinesterase was extracted from the fast extensor digitorum longus and slow soleus muscles of control, non-treated 6-week-diabetic and insulin-treated diabetic rats. The extracts were applied to velocity sedimentation and acetylcholinesterase activity was determined.
*Results.* We observed considerable differences in the distribution of individual acetylcholinesterase molecular forms in diabetic fast muscles. This included a 59 % decline in G4 content together with a fivefold increase in A8 and a 53 % increase in A12 activity resulting in a shift of acetylcholinesterase profile characteristically towards slow muscles. These alterations were partly reversed by insulin treatment.
*Conclusion/interpretation.* In slow muscles diabetes caused an increase in G4 activity without affecting the sedimentation profile. Decline in G4 content in fast muscles could contribute to enhanced desensitization of acetylcholine receptors in diabetes. [Diabetologia (2001) 44: 220–223]

**Keywords** Rat skeletal muscle, extensor digitorum longus, soleus, acetylcholinesterase, molecular forms, streptozotocin-diabetes, insulin treatment.

Acetylcholinesterase (AChE, E.C. 3.1.1.7) is a polymorphic enzyme and exists in globular (G1, G2, G4) and asymetric (A4, A8, A12) forms in skeletal muscles [1]. The proportion of the forms and the regulatory mechanisms vary according to the muscle type (fast-twitch or slow-twitch) [2]. The G4 and A12 forms play the most important part in neuromuscular transmission. In fast muscles the G4 form is regulated by nerve-derived signals, by pattern of trans-synaptic activation and by trophic factors [2, 3]. This form is found perijunctionally and prevents nicotinic acetylcholine receptor desensitization. The A12 form is the most abundant in the synaptic cleft in both muscle types and ends transmission [1].

Neuromuscular transmission is involved in diabetes mellitus, contributing to different alterations in the physiology of fast and slow skeletal muscles and leads to common clinical complaints of muscle weakness and fatigue [4]. A significant alteration in diabetes mellitus without separation of acetylcholinesterase molecular forms and differentiation between muscle types has not been found [5]. Distinction of muscle types seems, however, to be necessary owing to the known differences in content and regulation of molecular forms.

Our aim was to examine the effect of diabetes mellitus and insulin treatment on the acetylcholinesterase molecular forms extracted from fast extensor digitorum longus and slow soleus muscles.

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*Abbreviations:* AChE, Acetylcholinesterase; ACh, acetylcholine; ATCh, acetylthiocholine iodide; EDL, extensor digitorum longus; SOL, soleus; C, control; D6, 6 weeks diabetic; D6R3, 3 weeks diabetic + 3 weeks insulin-treated diabetic rats.

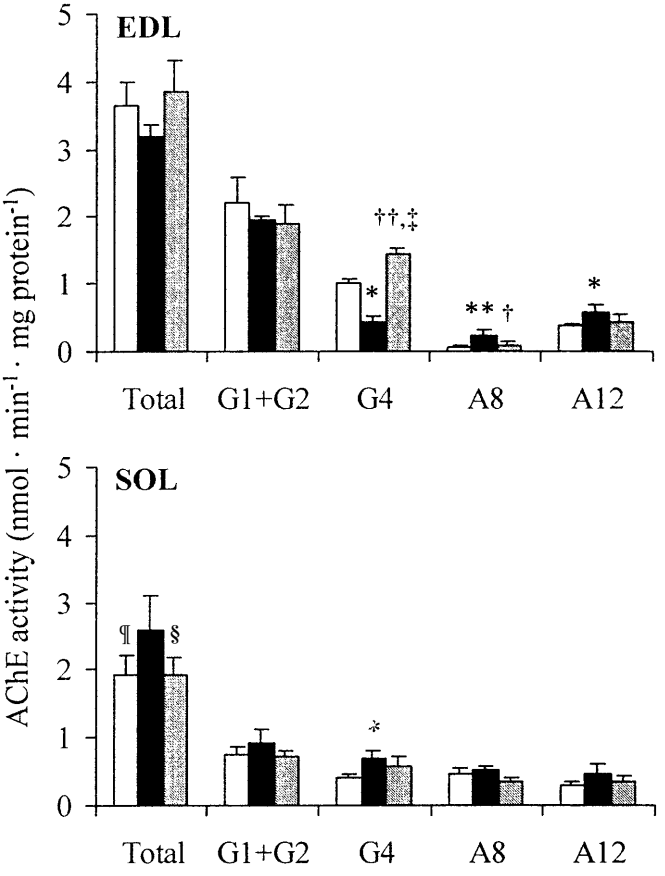


**Fig. 1.** Acetylcholinesterase (AChE) activity in extensor digitorum longus (EDL) and soleus (SOL) muscles. The enzyme activity was measured in control (white bars), streptozotocin-diabetic (black bars) and insulin-treated streptozotocin-diabetic (grey bars) muscles. Total enzyme activity of muscle extracts and activity of individual enzyme forms are shown. Activity of molecular forms was calculated from specific enzyme activity of muscles extracts and sedimentation profiles. Data are means ± SEM of 6–7 experiments. ¶ $p < 0.05$ EDL vs. SOL in control group. § $p < 0.05$ EDL vs SOL in insulin-treated streptozotocin-diabetic group. * $p < 0.05$ C vs D6. ** $p < 0.01$ C vs D6. † $p < 0.05$ D6 vs D6R3. †† $p < 0.01$ D6 vs D6R3. ‡ $p < 0.05$ C vs D6R3

## Materials and methods

*Materials.* Streptozotocin, bovine serum albumin (BSA), bacitracin, benzamidine, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 10-(2-diethylaminopropyl) phenothiazine hydrochloride (ethopropazine hydrochloride), was obtained from Sigma-Aldrich (Budapest, Hungary), acethylthiocholine iodide (ATCh) and insulin from Fluka (Buchs, Switzerland), blood glucose assay kits were from Boehringer (Mannheim, Germany), sucrose from Merck (Darmstadt, Germany).

*Animals.* Six week old, male Sprague Dawley rats (LATI, Gödöllõ, Hungary) weighing 190 to 260 g were randomly assigned to three groups: C (age matched controls), D6 (untreated diabetes for 6 weeks) and D6R3 (untreated diabetes for 3 weeks after insulin treatment for another 3 weeks). Diabetes was induced by a single injection of streptozotocin as described previously [6] and verified 48 h later by detecting hyperglycaemia (blood glucose level > 18 mmol/l) and glycosuria. In D6R3 group 4 IU Ultralente insulin was administered subcutaneously twice a day. Animals were anaesthesized and then killed by decapitation and trunk blood was collected to measure blood glucose. The EDL and SOL muscles were immediately removed, frozen in liquid nitrogen and stored at –80 °C.

*Extraction of AChE and velocity sedimentation analysis.* Muscle samples were homogenized in ice-cold high-salt detergent buffer and centrifuged (20000 · g, 15 min, 4 °C) [3]. We layered 25 µl of the supernatants on 2 ml 5 to 20 % (w/w) sucrose gradients and centrifuged in a Beckman TLS-55 rotor (32000 · rpm, 16 h, 4 °C). Catalase (sedimentation coefficient: 11.3 S) was used to estimate the sedimentation coefficients. Fractions were collected from the bottom of each gradient for AChE assays.

*AChE assay.* Enzyme activity was measured using Ellman's method [7] and carried out in a Bio-Tek FL600 microplate reader (Winooski, Vermont, USA) at 37 °C in 200 µl reaction mixture containing 50 mmol/l potassium phosphate buffer pH 7.0, 0.5 mmol/l DTNB, 0.01 mmol/l ethopropazine and 0.75 mmol/l ATCh. The breakdown of ATCh was measured by changes in the absorbency at 405 nm. The activity of different AChE forms was calculated by comparing the area under each peak to that under the entire sedimentation profile using the Origin 6.0 software (OringinLab Corp., Northampton, Mass., USA).

*Protein content.* Protein content was assayed by the Bradford method [8] using bovine serum albumin as standard.

*Statistical analysis.* Results are expressed as means ± SEM. Unpaired Student's *t*-test was used to compare groups. The difference between the means was considered to be significant, if $p$ was less than 0.05.

## Results

Streptozotocin-diabetic state increased plasma glucose concentrations from 8.3 ± 0.3 mmol/l to 21.7 ± 1.1 mmol/l ($p < 0.001$). Insulin treatment decreased this parameter to 5.7 ± 0.6 mmol/l ($p < 0.01$ vs D6) below the control parameter ($p < 0.05$ vs C). The absolute weight of EDL and SOL muscles was lower by 41 % and 33 %, respectively in D6 group ($p < 0.001$). Insulin treatment caused a partial recovery. Diabetes induced a significant ($p < 0.05$) 12 % decline in fast EDL muscle:body weight ratio without affecting the slow SOL muscle:body weight ratio. Insulin treatment led to complete recovery. There was a significant ($p < 0.05$) difference in total AChE activity between normal EDL (3.66 ± 0.33 nmol · min$^{-1}$ · mg protein$^{-1}$) and SOL (1.91 ± 0.29 nmol · min$^{-1}$ · mg protein$^{-1}$). Although diabetes did not cause a considerable change in total AChE activity of either muscles, due to the slight decrease in EDL (to 3.19 ± 0.16 nmol · min$^{-1}$ · mg protein$^{-1}$) and increase in SOL (to 2.58 ± 0.51 nmol · min$^{-1}$ · mg protein$^{-1}$) enzyme activities, there was no noteworthy difference found between the muscle types. Insulin

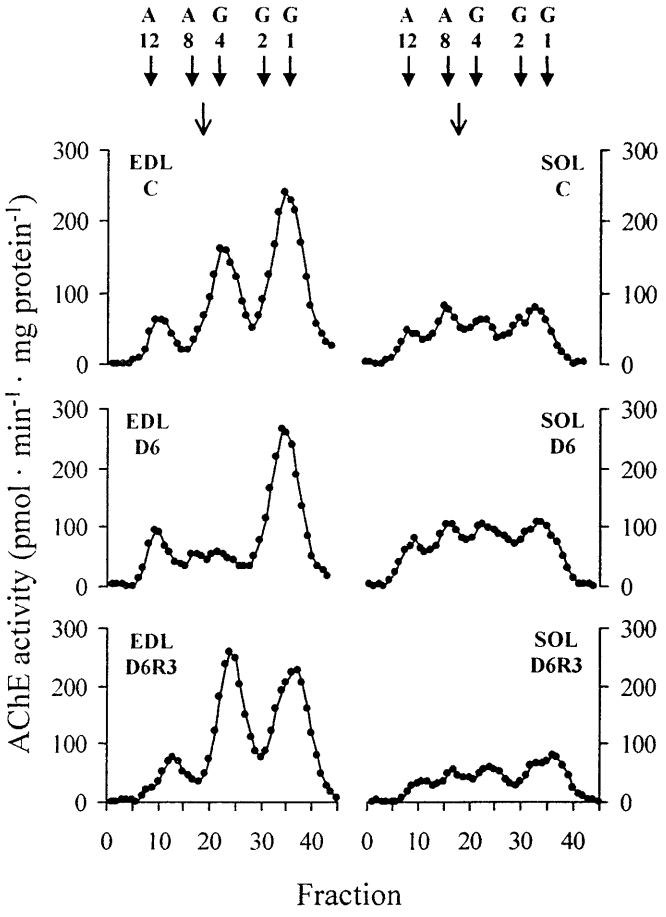


**Fig. 2.** Velocity sedimentation analysis of acetylcholinesterase (AChE) molecular forms observed in extensor digitorum longus (EDL) and soleus (SOL) muscles. Shown are representative examples of the average content of enzyme forms in control (C), streptozotocin-diabetic (D6) and insulin-treated streptozotocin-diabetic (D6R3) animals. The top of the gradients is on the right hand side. The open arrow indicates the position of catalase (sedimentation coefficient: 11.3 S) in the gradients

treatment normalized the activities: EDL: 3.85 ± 0.45 nmol · min$^{-1}$ · mg protein$^{-1}$ and SOL: 1.93 ± 0.25 nmol · min$^{-1}$ · mg protein$^{-1}$ and restored the significant difference ($p < 0.05$) between the two muscle types (Fig. 1).

After separation on sucrose gradients five peaks were identified by their known sedimentation coefficient [3] corresponding to the following forms: G1 (4 S), G2 (6.5 S), G4 (10 S), A8 (12.5 S) and A12 (16 S) (Fig. 2). The G1 and G2 forms were calculated together because they could not be separated consistently. Despite its small quantity, A8 could be detected by the curve fitting software in each group. In EDL muscles the specific activity of G1 + G2 forms did not change either in the diabetic condition or with insulin replacement.

The activity of the G4 form dropped considerably, by 59% ($p < 0.05$) in diabetes. Insulin treatment caused a significant increase ($p < 0.01$ vs D6) to 141% of control activity (it can be related to the lower blood glucose concentration).

A8 content increased about 5 times ($p < 0.01$) in the D6 group, whereas insulin treatment caused an almost total recovery. Similarly in the case of A12, diabetes resulted in a 53% ($p < 0.05$) augmentation. Because of the substantial increase in A8 and moderate rise in A12, the A8:A12 ratio increased in diabetes (Fig. 1). In SOL diabetes caused a rise in the content of each enzyme form. This increase was significant only in G4 (69%, $p < 0.05$) but the slight rise in the activity of other forms precluded a considerable change in the proportion of different forms. The acetylcholinesterase profile in diabetes remained similar to that seen in normal SOL (Fig. 1).

## Discussion

Several tissues show considerable differences in total AChE activity in diabetes. This reflects the dysfunction of the cholinergic system in this disease [9]. Other investigators did not find any alteration in enzyme activity in diaphragm muscles from diabetic mice [5]. After the separation of AChE molecular forms, we showed, however, striking alterations in AChE activity in EDL and SOL in diabetes. In healthy animals fast muscles show a typically high G4 activity, slow muscles contain less G4, and the A8:A12 ratio is higher. Diabetes produced a considerable shift in AChE profile of fast EDL towards that of slow-type muscles with a decreased G4 content and an increased A8:A12 proportion. The observed changes in fast muscles could be induced by altering neuromuscular activity or by altering the release of trophic factors.

In the soleus muscle the activity of the G4 AChE form increased remarkably in diabetes but according to our present knowledge this form does not play a specific part in slow muscles. Therefore diabetic SOL retained the AChE profile, which is characteristic of normal slow muscles.

The G4 and A12 AChE forms play the most important part in neuromuscular transmission [1]. Asymetric forms, mainly A12, are concentrated in synaptic basal lamina, where they are responsible for rapid hydrolysis of ACh released from motor nerve terminals [1]. These forms are present in an excessive amount [3] so that changes measured in diabetes do not necessarily have any functional consequences. The large changes observed in G4 content of diabetic fast muscles are, however, at a level that could have functional outcomes [2]. Therefore G4 AChE seems to be essential in diabetic dysfunction of fast muscles, because this form is supposed to have a special role in these muscles [3], namely preventing desensitization of nicotinic ACh receptors by maintaining a low background of ACh concentration. Diabetes is associated with accelerated desensitization of nicotinic

receptors [10], which can be partly explained by the lower G4 AChE content.

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