Loss of fast-twitch isomyosins in skeletal muscles of the diabetic rat

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1. By means of pyrophosphate electrophoresis the myosin isoenzyme pattern of two fast-twitch skeletal muscles (extensor digitorum longus, gastrocnemius) and one slow-twitch muscle (soleus) was investigated in control rats and was compared with that of rats 4 weeks after induction of diabetes mellitus by streptozotocin injection. 2. In the fast-twitch muscles the isomyosin pattern consisting of FM₁ (fast isomyosin 1), FM₂ and FM₃ was strongly affected by diabetes, resulting in an extensive loss of FM₁ and a substantial decrease of FM₂. 3. These changes were also apparent when the light chains of the fast isomyosins were analysed by two-dimensional electrophoresis: LC3f (myosin light chain 3f) largely disappeared and LC2f was significantly diminished. 4. In contrast, the isomyosin pattern in soleus muscle, consisting of SM₁ (slow isomyosin 1) and SM₂, was not affected by the diabetic state, and two-dimensional electrophoresis revealed a normal light-chain pattern of LC1sa, LC1sb and LC2s. 5. These results indicate that the isomyosins of slow-twitch oxidative myofibres are more resistant to the hormonal and metabolic disorders during diabetes mellitus than are the isomyosins of fast-twitch fibres.

The motility of the vertebrate organism is based on the myofibrillar proteins in the contractile apparatus of skeletal muscles. With respect to their mechanical and metabolic properties, myofibres can be classified into three different types: fast-twitch-glycolytic fibres (FG), fast-twitch-oxidative-glycolytic fibres (FOG) and slow-twitch-oxidative fibres (SO). Whereas the different extents of oxidative and glycolytic capacity in the three fibres is due to the amount and activities of the enzymes catalysing the biological oxidation and the glycolytic pathway, their different mechanical properties result, at least partly, from a differing contents of isoforms of the myofibrillar proteins. Fast and slow fibres not only differ in their relative content of α - and β -subunits of tropomyosin (Carraro *et al.*, 1981b), but also in their content of fast and slow type of troponin I (Dhoot & Perry, 1979) as well as in their slow and fast iso-

forms of myosin (Hoh & Yeoh, 1979). In chicken (Hoh *et al.*, 1976), in rat (d'Albis *et al.*, 1982) and man (Fitzsimons & Hoh, 1981), fast fibres irrespective of the glycolytic and oxidative capacity contain the three different isomyosins FM₁, FM₂ and FM₃, whereas slow fibres contain two different isomyosins, SM₁ and SM₂.

This characteristic pattern of contractile proteins in the muscle fibres is not only maintained by nervous supply (Carraro *et al.*, 1981a), but also by the well-balanced action of several hormones (Hoh *et al.*, 1978; Flink *et al.*, 1979; Johnson *et al.*, 1980; Dillmann, 1980), since thyroid hormone, growth hormone, glucocorticoids, catecholamines and, most importantly, insulin, are controlling the continuous synthesis and degradation of muscle proteins (Tischler, 1981).

A pathological deficiency of insulin, for instance, as it occurs in type I diabetes mellitus and as frequently investigated by experimentally induced diabetes mellitus in the rat, is characterized by a considerable disturbance of muscle protein turnover (Jefferson *et al.*, 1981); a decrease of protein synthesis (Pain & Garlick, 1974) and an increase in protein degradation (Garlick *et al.*, 1981) leads to a loss of muscle protein. Since elevated levels of 3-methylhistidine urinary excretion were

Abbreviations used: 2D electrophoresis, two-dimensional electrophoresis; EDL, extensor digitorum longus; FG, fast-glycolytic; FOG, fast-oxidative-glycolytic; FM, fast isomyosin; LC, myosin light chain; SDS, sodium dodecyl sulphate; SM, slow isomyosin; SO, slow-oxidative.

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found in rats after chemically induced diabetes mellitus (Dahlmann *et al.*, 1979; Nagasawa *et al.*, 1982), the general loss of muscle protein seems also to include a loss of myofibrillar proteins.

The present experiments were undertaken to study the pattern of isomyosins in various skeletal muscles in rat after streptozotocin-induced diabetes mellitus, and to test whether the entire spectrum or only discrete isoenzymes of myosin are affected by the diabetic state.

Experimental

Animals

Female wistar rats (Bor: WISW from Winkelmann, Borcheln, Germany) with a body weight of about 190 g were used. After overnight food deprivation they were divided into two groups. Diabetic rats: animals of this group were intravenously injected with 70 mg of streptozotocin/kg body wt. Streptozotocin (from Boehringer, Mannheim, Germany) was dissolved in 1 ml of 0.1 M-phosphate/citrate buffer, pH 4.5. Control rats: animals of this group received an intravenous injection of 1 ml of 0.1 M-phosphate/citrate buffer, pH 4.5. After the injections, all animals were given food and water *ad libitum*.

At the time that the diabetic animals were killed, blood glucose levels were 31.3 ± 3.4 mM ($n = 15$). These animals suffered from heavy glucosuria, polyphagia and polyuria. Mild ketonuria was observed only on the first day after streptozotocin injection. Control rats had blood glucose levels of 5 ± 0.7 mM ($n = 15$). At 4 weeks after induction of diabetes, all animals were killed and gastrocnemius, soleus and EDL muscles were excised and quickly frozen at -20°C .

Preparation of myosin

For preparation of myosin from soleus and EDL respectively, the left and right muscle of a rat were pooled. For myosin preparation of musculus gastrocnemius, only a single muscle was used. The muscles were homogenized in a 20-fold volume (v/w) of ice-cold 50 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4/25$ mM-KCl, pH 6.8, in an MSE homogenizer for 1 min at 14000 rev./min. The homogenate was centrifuged for 20 min at 12000 g (4°C). The pellet was resuspended in 2–5 ml of 100 mM- $\text{Na}_4\text{P}_2\text{O}_7/1$ mM-dithioerythritol/1 mM-EDTA, pH 8.8, and after being left for 60 min at 4°C , the homogenate was again centrifuged for 20 min at 12000 g (4°C). The final supernatant was directly used for electrophoretic analysis of myosin isoenzymes and light-chain content.

Electrophoretic techniques

All reagents used for preparation of polyacrylamide gels were from Serva, Heidelberg, Germany,

except for SDS, which was from Pierce Chemical Co., Rockford, IL., U.S.A. Pyrophosphate electrophoresis of native myosin was carried out as described by Hoh *et al.* (1976) as modified by d'Albis *et al.* (1979). Briefly, cylindrical polyacrylamide gels (4 mm $\times$ 100 mm) containing 3.88% (w/v) acrylamide and 0.12% (w/v) methylenebisacrylamide in 40 mM- $\text{Na}_4\text{P}_2\text{O}_7/0.4\%$ NNN'-tetramethylethylenediamine/1 mM-EDTA, pH 8.5, were polymerized by addition of 0.2% (w/v) ammonium persulphate. The electrophoresis buffer was 40 mM- $\text{Na}_4\text{P}_2\text{O}_7/10\%$ (v/v) glycerol/1 mM-dithioerythritol/1 mM-EDTA, pH 8.8. After a pre-run of 30 min, myosin samples [$10 \mu\text{g}$ in $35 \mu\text{l}$ containing 50% (v/v) glycerol] were electrophoresed for 18 h at 3°C with a constant voltage gradient of 10 V/cm. Electrophoresis buffer was constantly recirculated between the lower and the upper reservoir.

After the run the gels were fixed in a solution of 10% (w/v) trichloroacetic acid/50% (v/v) methanol for 60 min and then stained with 0.03% (w/v) Coomassie R250 dissolved in propan-2-ol/acetic acid/water (5:2:13, by vol.) for 60 min. Destaining was performed in acetic acid/methanol/water (3:20:17, by vol.) for 60 min before the gels were stored in 7.5% (v/v) acetic acid.

2D polyacrylamide-gel electrophoresis was carried out as follows. Cylindrical polyacrylamide gels for isoelectric focusing (first dimension) were prepared as described by O'Farrell (1975), except that the gels contained 3% (v/v, final concn.) ampholyte consisting of 80% (v/v) Servalyte pH 5–7, and 20% (v/v) Servalyte pH 3–7. Electrophoresis buffer was 20 mM-histidine (anode) and 20 mM-taurine (cathode). Electrophoresis was run until 6500 Vh were reached with a maximum voltage gradient of 1000 V.

After removal of the gels from the tube, they were equilibrated in 2% (w/v) SDS/50 mM-Tris/HCl/10 mM-dithioerythritol, pH 7.5, for 60 min at 21°C , before being polymerized with 1% (w/v) agarose on top of the SDS/polyacrylamide slab gel (second dimension).

SDS/polyacrylamide slab gels were prepared exactly as described by Chua (1980). The gels contained a polyacrylamide gradient of 10–18% (w/v) and were run at 12 mA constant current for 18 h.

Staining of the slab gels was performed with 0.2% (w/v) Coomassie R250 in acetic acid/methanol/water (7:40:53, by vol.) for 60 min and destaining in 7.5% (v/v) acetic acid.

Densitometric scanning of the stained polyacrylamide gels of pyrophosphate electrophoresis and calculation of the area of the peaks was performed with an 2202 Ultrosan laser densitometer equipped with an integrator (LKB, Bromma, Sweden).

For determination of protein concentration the method of Bradford (1976) was used, with control serum (LAB TROL; Dade, Miami, FL, U.S.A.) as a standard.

Results

Soleus muscle

Soleus muscle is a red, tonic muscle containing 84% SO fibres and only 16% FOG fibres (Ariano *et al.*, 1973). Resolution of soleus-muscle myosin isoenzymes by pyrophosphate electrophoresis showed two protein bands, representing SM₁ and SM₂, with the latter as the predominant isoform (Fig. 1a; Table 1). Analysis of light chains of these isoenzymes by 2D-electrophoresis revealed the typical three-spot pattern of the slow-twitch myosin light chains: LC1sb, LC2s, and the LC1sa detectable in the more alkaline pH range after isoelectric focusing (Fig. 2a).

At 4 weeks after induction of diabetes mellitus neither the isoenzyme pattern as analysed by pyrophosphate electrophoresis (Fig. 1b; Table 1) nor the myosin-light-chain pattern as analysed in 2D-electrophoresis was different from the pattern in the control soleus muscle (Fig. 2b).

EDL muscle

Rat EDL muscle is composed of 59% FOG, 38% FG and only 3% SO fibres and has fast-twitch contraction properties (Ariano *et al.*, 1973). The isomyosin spectrum consists of about equal amounts of the two isoenzymes FM₁ and FM₂ and a smaller

quantity of FM₃ (Fig. 1c; Table 1), and the myosin-light-chain pattern is composed of LC1f, LC2f and LC3f (Fig. 2c).

At 4 weeks after onset of diabetes mellitus the isomyosin FM₁ is almost completely lost and the amount of FM₂ is diminished. Concomitantly, an isoenzyme with the mobility of SM₂ becomes apparent in pyrophosphate-electrophoresis gels (Fig. 1d; Table 1). These changes are paralleled by a considerable decrease in the amount of light-chain LCf3 and a relative increase in the amount of LC1sb and LC2s (Fig. 2d).

Table 1. Percentage distribution of fast and slow isomyosins in soleus, EDL and gastrocnemius muscles from control and diabetic rats

The relative amounts of myosin isoenzymes after their resolution by pyrophosphate electrophoresis was measured by scanning the gels shown in Fig. 1 and integrating the area under the absorbance peaks.

Muscle	Percentage of total myosin				
	Isomyosin ...	SM ₁	SM ₂	FM ₁	FM ₂ FM ₃
Soleus					
Control		11.9	88.1		
Diabetic		9.3	90.7		
EDL					
Control				37.2	39.8 23.0
Diabetic			2.4	6.1	29.6 61.9
Gastrocnemius					
Control			2.9	30.8	37.4 28.9
Diabetic			10.3	12.2	26.1 51.4

Fig. 1. Densitometric scans of pyrophosphate/polyacrylamide gels from electrophoresis of myosins from individual rat skeletal muscles

(a) soleus, control rat; (b) soleus, diabetic rat; (c) EDL, control rat; (d) EDL, diabetic rat; (e) gastrocnemius, control rat; (f) gastrocnemius, diabetic rat. The positions of the isoenzymes of slow myosin (SM₁, SM₂) and of fast myosin (FM₁, FM₂, FM₃) are indicated.

Fig. 2. 2D polyacrylamide-gel electrophoresis of myosins from individual rat skeletal muscles. The first (isoelectric focusing) and second (SDS/polyacrylamide-gel electrophoresis) dimensions are indicated. Only the light-chain region, including actin (ac) and tropomyosin (tm) spots, are shown. (a) soleus, control rat; (b) soleus, diabetic rat; (c) EDL, control rat; (d) EDL, diabetic rat; (e) gastrocnemius, control rat; (f) gastrocnemius, diabetic rat. The positions of the slow myosin light chains (LC1sa, LC1sb, LC2s) and of the fast-myosin light chains (LC1f, LC2f, LC3f) are indicated.

Gastrocnemius muscle

Similar in composition to EDL, gastrocnemius muscle is composed of 95% fast-twitch fibres and 5% slow-twitch fibres (Ariano *et al.*, 1973). However, in contrast with EDL, this muscle contains a higher percentage of FG fibres (58%) than FOG fibres (37%). This difference, however, is not reflected in the myosin isoenzyme pattern of the two muscles, since the relative amounts of FM₁, FM₂ and FM₃ in gastrocnemius are similar to those measured in EDL muscle (Fig. 1e; Table 1). The light-chain pattern consists of LC1f, LC2f and LC3f, together with low amounts of the slow-twitch components LC1sb and LC2s (Fig. 2e), due to the presence of trace amounts of SM₂ (Fig. 1e; Table 1).

Again, diabetes mellitus results in a considerable decrease of fast-twitch isomyosin FM₁ and FM₂ and a significant increase in the amount of SM₂ (Fig. 1f; Table 1). Accordingly, the amounts of LC3f and also LC2f are diminished in the diabetic muscle, whereas LC2s, and especially LC1sb, are now clearly detectable (Fig. 2f).

Discussion

The myosin isoenzyme pattern of slow-twitch soleus and fast-twitch EDL and gastrocnemius muscles in normal rat found in our sodium pyrophosphate-electrophoresis experiments are identical with the results previously reported by others (d'Albis *et al.*, 1982; Carraro *et al.*, 1982). Extending these findings our experiments clearly indicated that 4 weeks after induction of diabetes mellitus a partial or complete loss of the fast isomyosins FM₁ and FM₂ had occurred in gastrocnemius and EDL muscle, whereas the isomyosins of the SO fibres were not affected or had slightly increased in concentration. These data obtained by electrophoresis of native myosin are supported by the results of the light-chain analysis by means of 2D-electrophoresis. According to Hoh and co-workers the fast isomyosins have the following light chain composition: FM₁ (LC2f)₂ (LC3f)₂; FM₂ (LC1f) (LC2f)₂ (LC3f)₂; FM₃ (LC1f)₂ (LC2f)₂ (Hoh, 1978; Hoh & Yeoh, 1979). Thus a loss of FM₁ in diabetic gastrocnemius and EDL muscles should predominantly result in a decline of LC3f, since 2 mol of the light chain are contained in 1 mol of this isoenzyme. This is demonstrated by the results shown in Fig. 2d and 2f. Additionally, the decline of FM₂ in these muscles amplifies the decrease of LC3f and, altogether, result also in a diminution of LC2f.

Further, owing to the slight increase in SM₂ isomyosin in diabetic EDL and gastrocnemius muscle, an intensification of the spots of LC1sa,

LC1sb and also LC2s in the 2D electrophoresis was apparent. Whether this increase in slow-twitch isomyosin is due to an enhanced synthesis of SM₂ or only a relative increase with respect to the decreasing amount of fast isomyosin cannot be decided from our experiments. However, since in plantaris muscle of chronic diabetic rats both the percentage of SO fibres and the SO fibre diameter are unchanged, whereas a significant decrease in FOG- and FG-fibre diameter can be measured (Armstrong & Ianuzzo, 1977), it is very likely that the increase we observed in SM₂ content in EDL and gastrocnemius muscle is also only a relative one.

These results, together with the observation that the isomyosin pattern in soleus muscle has not visibly changed 4 weeks after induction of diabetes, clearly demonstrate, that the slow-twitch myosin SM₁ and SM₂ and, more generally, the SO fibres, are more resistant to diabetic conditions than are fast-twitch fibres. These findings are in complete accordance with the finding that diabetes induced by alloxan injection has a more pronounced effect on the protein-synthesis rate in fast-twitch myofibres than in slow-twitch fibres: a decrease of 40–50% was observed in FOG and FG fibres, whereas in SO fibres a reduction in protein synthesis of only 20% was measured (Flaim *et al.*, 1980). Further, using histochemical methods, Armstrong *et al.* (1975) observed a general de-differentiation and a decrease of fibre area in FOG and FG fibres in diabetic-rat gastrocnemius muscle, together with a general loss of oxidative potential (decrease of NADH diaphorase staining) in these fibres, which is paralleled by a decreased number of mitochondria in FOG and FG fibres (Chao *et al.*, 1976). On the other hand, neither a loss of oxidative capacity nor extensive morphological alterations were observed in muscles, like soleus muscle, containing predominantly SO fibres.

Since fast-twitch isomyosins have a higher myosin ATPase activity than have slow-twitch myosins (d'Albis *et al.*, 1982; Unsworth *et al.*, 1982), a loss of fast-twitch isomyosins during the diabetic condition should result in a decrease of myosin ATPase activity. This has indeed been found in a previous study from this laboratory (Röthig *et al.*, 1978). In heart muscle, also, the myosin ATPase activity is diminished during the diabetic condition, and this effect is also accompanied by alterations in the myosin isoenzyme pattern (Dillmann, 1980).

From the present results the question arises as to why exactly these two fast-twitch isoenzymes FM₁ and FM₂ are affected, and the other, FM₃, as well as the two slow-twitch isomyosins, are not. A further question remaining to be answered is whether

the loss of the fast-twitch isomyosins results from a decreased synthesis of LC3f and/or of one of the two fast-twitch myosin heavy-chain isoforms (Zweig, 1981; Billeter *et al.*, 1982) and/or results from an increased breakdown of these components.

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