A New Electrophoretic Micromethod for Assessing Myosin Heavy Chain Composition of Skeletal Muscle in Chronic Heart Failure

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Abstract

Chronic heart failure is a clinical disorder characterized by exercise intolerance. Fatigue and shortness of breath are the symptoms causing a patient to stop exercising. It is clear from previous studies that in this syndrome the skeletal muscle of the lower limbs develops a myopathy with atrophy and shift from the slow type to the fast type fibres.

We devised a new electrophoretic micromethod that is able to separate, from 50-150 µg needle biopsies of the gastrocnemius muscle, the three different isoforms of Myosin Heavy Chains (MHC). A 7.5% polyacrylamide SDS PAGE with 37.5% glycerol was used. About 1 µg of protein was loaded on each well and silver stain was used to detect the isolated isoforms. The percentage of MHC1 (slow isoform), MHC2A (fast oxidative) and MHC2B (fast glycolytic) was determined by densitometric scan. We studied 19 patients with chronic heart failure (age 71.2 ± 7.6) whose ejection fraction was 42.5 ± 15.2. MHC1 was 51.83 ± 15.04 whilst MHC2B was 23.5 ± 7.4 and MHC2A 24.83 ± 15.01. Within the CHF group there was a positive correlation between NYHA class and MHC2A ($r = 0.47$, $p < 0.05$) and MHC2B ($r = 0.55$, $p < 0.01$) and a negative correlation between NYHA class and MHC1 ($r = 0.74$, $p < 0.001$). Similarly, significant correlations were found for ejection fraction, diuretic consumption score, exercise test tolerance and degree of muscle atrophy.

These data seem to suggest the hypothesis that the myopathy present in patients with chronic heart failure is specific and that the severity of the disease correlate with the magnitude of the MCH redistribution.

The micromethod used in this study to evaluate MHC isoforms seems to be very reproducible and well tolerated by the patients so that it allows to take several biopsies and therefore enabling thorough follow up.

Key words: myosin heavy chain, heart failure, skeletal muscle, electrophoresis.

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Chronic Heart Failure (CHF) is a clinical disorder characterized by exercise intolerance. Fatigue or shortness of breath are the symptoms causing a patient to stop exercising. It has been shown that lower limbs skeletal muscle performance is disordered in CHF and this can at least in part explain symptoms complained by the patient [21]. Lipkin and Poole-Wilson [17] initially suggested that *quadriceps femoris* muscle strength is reduced because of loss of skeletal muscle bulk, although the mean force per unit area was within the normal range [21, 22].

Muscle wasting in CHF is accompanied by profound histologic changes. Lipkin et al. [17] reported results of the quadriceps muscle biopsies showing a shift towards type II fibres with presence of atrophic fibres. Mancini et al. [20] studied the *gastrocnemius* reporting a shift toward type IIB fibres and atrophy of the type IIA. Sullivan and Cobb [25] examined needle biopsies from *vastus lateralis* and found type I fibres reduced and IIB to be increased. Drexler et al. [10] looked into the possible contribution of mitochondrial abnormalities to the myopathy of CHF. In

the *vastus lateralis* they found a reduction in volume density of mitochondria and surface density of christae. They also found a reduction of type I and a relative increase of type II fibres.

We tried to correlate the severity of the disease with the magnitude of the shift in MHC pattern. We did that by means of a new electrophoretic micromethod devised in our laboratories and able to separate the three skeletal muscle MHC isoforms, namely the slow MHC1 isomyosin, the MHC2A (fast oxidative) and the MHC2B (fast glycolytic).

Patients and Methods

Patients

Nineteen patients with CHF ((10 males, 9 females) (9 with Dilated Cardiomyopathy (DCM), 4 with Hypertensive Heart Disease (HHD) and 6 with Ischaemic Heart Disease (IHD)) were studied. These were all patients with previous established diagnosis done by means of clinical history, ECG, exercise test, echo and coronary angiogram. Nine control patients were also studied.

Skeletal muscle biopsy

Skeletal muscle biopsies were taken from the *gastrocnemius* muscle by using a 17G Histo-Cut soft tissue disposable Menghini needle (Sterylab Inc. Milan, Italy). From each specimen between 50 and 150 μg of tissue were obtained. The specimen was immediately frozen in liquid nitrogen and kept in a -80°C fridge until processed. No anaesthesia was employed.

Exercise test tolerance

All the patients with CHF and the normal subjects underwent a treadmill exercise test according to a modified Naughton protocol. The protocol envisaged seven three minutes each stages at a fixed speed of 3 Km/h starting from 0% grade (stage 0) with increments of 3.5%. All subjects exercised to a symptom limited maximum or until they reached theoretical submaximal heart rate.

Echocardiographic measurements

All the CHF and control subjects had a 2D-Echo and color-Doppler. Ejection Fraction (EF), Left Ventricle End Diastolic Volume (LVEDV), Left Ventricle End Systolic Volume (LVESV), A wave/E wave ratio were calculated with a parasternal short axis approach. EF was calculated with the modified Simpson Method.

NYHA class

NYHA class was assessed in all the patients.

Diuretic consumption

Patients, according to Harding et al. [14] were divided into 5 classes according to diuretic consumption. Class 1 was no diuretic, class 2 20 to 40 mg of frusemide or a thiazide diuretic a day, class 3 ≥ 40 mg frusemide, class 4 ≥ 80 mg, class 5 ≥ 120 mg.

Electrophoretic separation of MHC

The method described by Carraro et al. was used [3, 23]. The muscle biopsies were solubilized in a small volume (100-200 μl) of 2.3% SDS, 10% glycerol, 0.5% 2-mercaptoethanol, 62.5 mM TRIS-HCl pH = 6.8 and boiled for 5 minutes. The running buffer was added to the sample in a ratio of 1:1 v/v. Analytical SDS page of MHC was performed on 7% polyacrylamide slabs, 0.75 x 130 x 130 mm. Slabs were prepared according to Laemmli [15], but 37.5% v/v glycerol was present both in the separating and in the stacking gel. The stacking gel was composed of 37.5% glycerol, 4% T acrylamyde-Bis (36.5:1), 12.5 mM TRIS-HCl (pH = 6.8), 0.1% SDS. The separating gel was composed of 37.5% glycerol, 7% T acrylamyde-Bis (36.5:1), 375 mM TRIS-HCl (pH = 8.8), 0.1% SDS. Running buffer was made of 50 mM TRIS, 384 mM glycine, pH = 8.3, 0.2% SDS (without correction of pH with HCl). Separation of MHC was achieved using constant current at 4 mA (corresponding to about 40 V). After 6-8 hours the running buffer was changed and electrophoresis restarted with the same parameters. After 24 hours the voltage rises to about 130-160 V and the run is stopped. Gels containing approximatively 0.2 μg of protein per band are stained with 0.1% Coomassie brilliant blu in 5% acetic acid, 40% methanol and destained with 40% methanol, 7% acetic acid. Gels with less than 0.1 μg protein were stained with Silver method. In a previous series of experiments [23] MHCs after SDS-page were immunoblotted with anti MHC1-2A mAb (BF32) and anti- MHC2X-2B (D9) demonstrating the good reproducibility of MHC separation.

Assessment of MHC content

The percent distribution of MHC1, MHC2A and MHC2B was determined by densitometric scan of the slab gel after staining with Coomassie brilliant blue. The scan was performed by using a GS300 Transmittance Reflectance Scanning Densitometer (Hoefer Scientific Instruments) connected to a McIntosh SE Apple Computer. The data were processed by using the GS370 Densitometry Software.

Statistical analysis

Coefficient of variation, and linear regression were used when appropriate.

Results

Age

Age was 71.2 ± 7.6 in the CHF group and 55.4 ± 8.5 in the controls (p = NS).

NYHA class

Between CHF patients 4 were in NYHA class I, 4 in NYHA II, 8 in NYHA III and 3 in NYHA IV.

Haemodynamic parameters

EF was 42.5% ± 15.2 (n = 19) in CHF patients and 60.3 ± 1.4 in controls (p < 0.001).

Diuretic consumption

Between CHF patients diuretic consumption score was 1 in 9 subject, 2 in 7, 3 in 6, 4 in 4 and 5 in 2.

Myosin Heavy Chain composition (MHC)

Myosin Heavy Chain composition (MHC) was 51.83 ± 15.04 for MHC1, 24.83 ± 15.01 for MHC2A and 23.5 ± 7.45 for MHC2B in the CHF group.

Correlation between MCH composition and parameters of clinical severity of CHF

Clinical parameters indicating the severity of heart failure were correlated with MHC composition in patients with CHF and controls.

a) NYHA class

There was a significant, negative correlation between percentage of MHC1 and NYHA class ($r = 0.74$, $p < 0.001$), whilst there was a positive correlation between NYHA class and MHC2A ($r = 0.47$, $p < 0.05$) and MHC2B ($r = 0.55$, $p < 0.01$) (Fig. 1).

b) Ejection Fraction (EF)

EF correlated conversely in significant, positive manner with MHC1 ($r = 0.65$, $p < 0.001$) and in a negative fashion with MHC2A ($r = 0.75$, $p < 0.001$). Although there was a trend for MHC2B to decrease in patients with higher EF, this did not reach statistical significance ($r = 0.26$, $p = NS$) (Fig. 2).

c) Diuretic consumption

Diuretic consumption score correlated significantly in a negative manner with MHC1 ($r = 0.62$, $p < 0.001$) and in a positive one with MHC2A ($r = 0.35$, $p < 0.1$) and MHC 2B ($r = 0.49$, $p < 0.001$) (Fig. 3).

d) Exercise Test Tolerance (ETT)

There was a significant correlation between steps reached on the treadmill and MHC composition. The correlation was in fact positive with MHC1 ($r = 0.65$, $p < 0.001$) and negative with MHC2A ($r = 0.37$, $p < 0.05$) and MHC 2B ($r = 0.57$, $p < 0.01$) (Fig. 4).

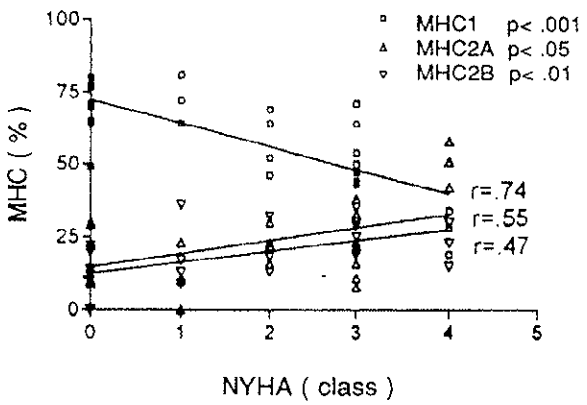

Figure 1. Correlation between Myosin Heavy Chain (MHC) composition and NYHA class.

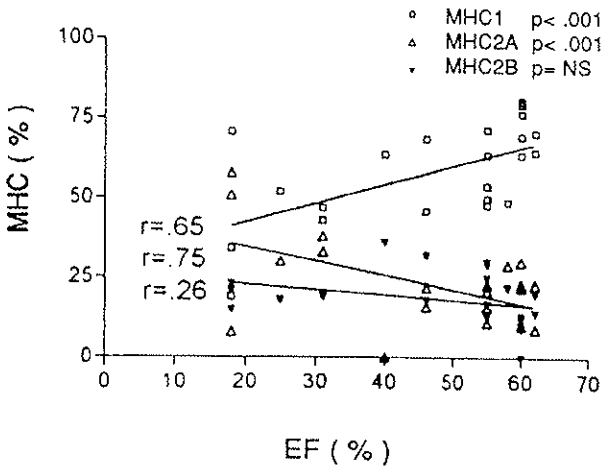

Figure 2. Correlation between Myosin Heavy Chain (MHC) composition and Ejection Fraction (EF).

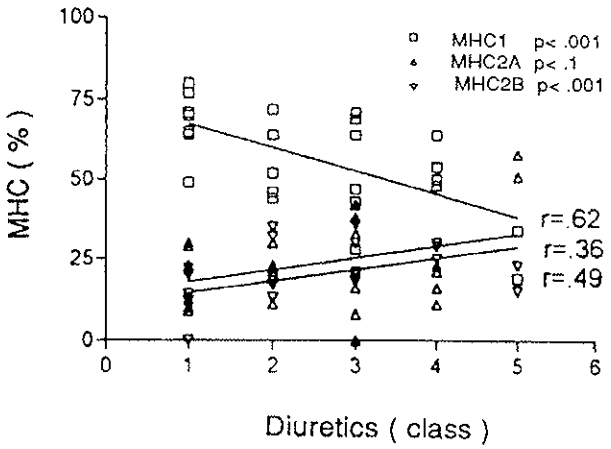

Figure 3. Correlation between Myosin Heavy Chain (MHC) composition and Diuretic Consumption.

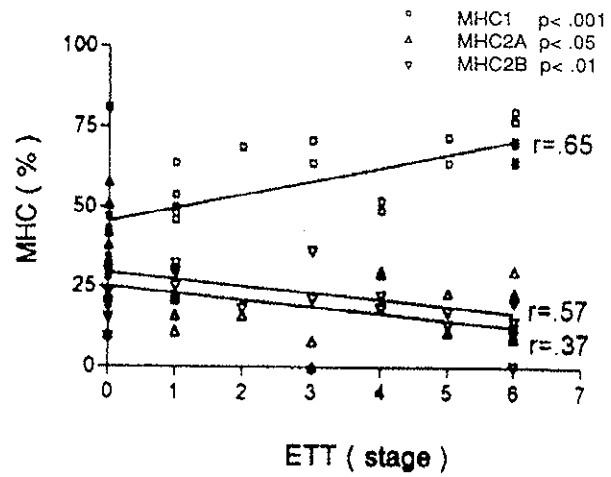

Figure 4. Correlation between Myosin Heavy Chain (MHC) composition and Exercise Test Tolerance (ETT).

Reproducibility of the method

Five biopsies were taken from the same subject with CHF and processed separately. On the same slab gel samples from the 5 different biopsies were loaded. The coefficient of variation between the 5 samples was 5% for MHC1, 5% for MHC2A and 7% for MHC2B.

We also loaded on 5 separate SDS-pages samples coming from one patient. Electrophoresis were carried out on separate days. The sample, once solubilized in SDS, 2-mercaptoethanol and TRIS-HCl, was kept in a fridge at -10°C until the experiment was done. The coefficient of variation was 4% for MHC1, 6% for MHC2A and 4% for MHC2B.

Discussion

The novelty of this study is the method we used for separating myosin heavy chains. It allows separation of the three isoforms from extremely small tissue samples. The reproducibility of the method is fairly high, in fact the coefficient of variation of MHC percent distribution from different biopsies from the same patient is low, as well as that found for the repeated Electrophoresis of the same bioptic sample.

We found a correlation between indicators of severity of CHF and MHC composition. We observed in fact a significant correlation between NYHA class, exercise tolerance, diuretic consumption and ejection fraction with the percentage of MHC isoforms. It looks as if patients with CHF have a specific myopathy the severity of which is related to that of the heart failure syndrome. Our data show that in patients with cardiac failure there is a shift toward the fast isoforms MHC2A and MHC2B, with a significant decrease of the slow isoform MHC1. In fact in a study where CHF patients were compared to normal subjects we found significant differences between these two groups of patients [28]. Our data are in agreement with those of Poole-Wilson [17], Mancini [20] and Sullivan [25] who described an increased percentage of easily fatigable, fast twitch IIB fibres and atrophy of type I slow twitch fibres. Similar results have been reported by Sabbah et al. [24] in dogs with chronic heart failure. These changes have been ascribed to intrinsic skeletal muscle abnormalities [8] and only in part to muscle underperfusion [21]. Moreover Wilson et al. [29] suggested that exertional fatigue is due to skeletal muscle abnormalities and not to reduced blood flow. Atrophy due to muscle deconditioning (disuse hypothesis) [9, 12, 27] has been claimed to be partially responsible for these muscle alterations. In term of the existence of a specific skeletal muscle myopathy with selective type I fibres atrophy, this has been reported by Caforio et al. [2] in patients with cardiomyopathies. These changes resamble alterations found in congenital and idiopathic myopathies but were found by these Authors to be unrelated to NYHA class. Conversely Dunnigan et al. [11] in patients with cardiomyopathy described a type II skeletal muscle fibre atrophy. Changes in skeletal muscle in CHF are very much the same of those observed in the muscle denervation in the rat [5]. In this experimental model in fact denervation is accompanied by an increased expression of adult fast myosins [5]. The synthesis of adult slow myosins is dependent on the actual activity of motor innervation, while adult fast is expressed in denervated fibres even though the neuronal influences are removed [1, 4]. This hypothesis is supported by observations in paraplegic patients where a slow to fast transformation occurs [13, 18]. Other Authors suggest different mechanisms. For instance, circulating factors such as increased concentrations of Tumor Necrosis Factor [19] or metabolic and vascular alterations like insulin resistance [26] or increased thickness of the endothelial cells [16] can be invoked as determinants of muscle changes. However a "neural theory" has been recently put forward to explain symptoms and muscle waste in CHF. Coats et al. [7] have in fact proposed the "ergo- and metabolo- receptors" theory, which envisages the existence of chemically sensitive receptors able to pick up muscle work and metabolism. They transmit to the central nervous system via small unmyelinated nerve fibres stimuli that can drive blood pressure responses and increase sympathetic activity. An increased ergoreceptor activity in patients with CHF might maintain the increased sympathetic outflow with consequent increased peripheral resistances, decreased perfusion and further muscle deterioration.

Fine needle biopsy is extremely easy to be performed. It does not cause any harm to the patient and is so well tolerated that does not require any sort of anaesthesia. We did not have any sort of complication in our series of patients. The technique allows serial bioptic sampling in the same patients enabling us to study either muscle worsening during the progression of the disease or possible improvements due to different therapeutic approaches such as training or hormonal therapy that seem able to restore muscle bulk, relief symptoms and increase exercise tolerance [6]. The study of MHC composition may also have the potential to detect early changes in skeletal muscle composition and correlate them with humoral, biochemical and functional parameters.

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