Mitochondrial function in heart muscle from patients with idiopathic dilated cardiomyopathy

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Abstract

Objective: To study the mitochondrial respiratory chain enzyme activities in patients with idiopathic dilated cardiomyopathy (IDC).

Methods: Mitochondrial respiratory chain enzyme activities were assessed spectrophotometrically in left ventricular tissue of 17 patients with IDC undergoing cardiac transplantation, as well as in two groups of controls: a group of six patients suffering from ischemic dilated cardiomyopathy (IC) also undergoing cardiac transplantation, and a group of 17 organ donors considered normal from a cardiac point of view. Cytochrome \(b\) gene from three IDC patients whose complex III activity was particularly low and from three controls was also sequenced.

Results: We found that complex III enzymatic activity was lower not only in IDC but also in IC patients when compared with normal controls. When analysing cytochrome \(b\) gene we only found neutral polymorphisms previously described.

Conclusions: In view of such results, we believe that the decrease of respiratory chain complex III activity found in some cases of IDC is a secondary phenomenon, and not due to a primary mitochondrial disease. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Idiopathic dilated cardiomyopathy (IDC) is a severe condition that results in heart enlargement and impairment of the ventricular contractile function. It causes progressive congestive heart failure that, in some patients, eventually requires cardiac transplantation. The precise mechanisms leading to ventricular dilatation and dysfunction are not well understood; however, during the last few years attention has been focused on abnormalities of contractile and structural myocardial proteins, and on disorders of cardiac energy metabolism [1].

Mitochondrial oxidative phosphorylation (OXPHOS) is essential to provide the energy for cardiac function. The OXPHOS system essentially comprises the electron transport chain (complexes I to IV) and the \(F_1F_0\) ATP synthase (complex V). These five protein complexes, located in the inner mitochondrial membrane, are constituted by a multitude of subunits, whose genes are distributed over both nuclear and mitochondrial DNA (mtDNA).

Recently, mtDNA defects have been found in an increasing number of cases of IDC. These abnormalities include the presence of specific point mutations in mitochondrial tRNAs (mtRNAs) and structural genes, as well as single large or multiple deletions [2–6]. Although in some cases a correlation between molecular defects and a decrease in specific mitochondrial enzymatic activities has

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been found, few studies focused on the characterisation of the respiratory chain function and the results have often been contradictory. This could be due, in part, to the difficulties in interpreting enzymatic results on autopsied hearts, or on the small fragments of myocardial tissue that can be obtained by means of heart biopsy. Moreover, the question of whether alterations in mtDNA sequence and determined according to Bradford [9].

The objective of the present study was to assess electron transport chain function in mitochondria from cardiac muscle of patients suffering from IDC undergoing heart transplant, and compare results with a group of patients with ischemic dilated cardiomyopathy (IC) and a group of normal (organ donor) controls.

2. Methods

2.1. Patients and samples

A total of 17 patients with IDC undergoing cardiac transplantation (12 men and five women, ages 38 to 63 years) were studied. Control groups were six patients with ischemic heart disease (six men, ages 51 to 60 years) also undergoing cardiac transplantation because of cardiac failure, and 17 organ donors (12 men and five women, ages 29 to 77 years) who died due to craneoencephalic traumatism or stroke, who had no history of heart disease, and whose heart was not suitable for transplantation, mainly due to lack of an appropriate receptor.

All patients undergoing cardiac transplantation were in heart failure class IV of the New York Heart Association (NYHA) classification. Patients or their families gave written consent, the protocol was approved by the ethical committee of our Institution, and conforms with the principles outlined in the Declaration of Helsinki.

Ventricular transmural biopsies were obtained from the explanted heart during cardiac transplantation. Samples weighting 3–5 g were collected on phosphate buffer saline and immediately stored in liquid nitrogen until further analysis.

2.2. Spectrophotometrical studies

Homogenates from frozen heart muscle specimens were prepared in 30:1 vol of 0.25 M sucrose, 40 mM potassium chloride, 2 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid, 1 mg/ml bovine serum albumin (BSA) and 20 mM Tris–HCl (pH 7.2). Tissues were disrupted by seven strokes at 800 rpm in a glass–PTFE potter. The homogenate was centrifuged at 2000 g for 8 min. The pellet was discarded and the supernatant was used for biochemical studies. The measurement of the specific activity of the individual complexes of the respiratory chain was performed spectrophotometrically (UVIKON 922 Spectrophotometer, Kontron, Switzerland). A total of 5–15 μg of protein was used to determine the activity of each complex, except for complex IV, for which we used 2.5 μg of protein. Assays were performed at 37°C in 1 ml of medium according to Rustin et al. [7] and Chretien et al. [8]. Protein was determined according to Bradford [9].

2.2.1. Measurement of the rotenone-sensitive NADH-decylubiquinone oxidoreductase (complex I)

The assay was performed at 340 nm using the acceptor 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (80 μM) and 200 μM NADH as donor, in 10 mM Tris (pH 8.0) buffer containing 1 mg/ml BSA, 0.24 mM KCN and 0.4 μM antimycin for 5 min. The addition of 4 μM rotenone allowed us to quantify the rotenone-sensitive activity. To permeabilize the mitochondrial internal membrane to NADH, sample was incubated in distilled H2O for 3 min at 37°C.

2.2.2. Measurement of succinate decylubiquinone 2,6-dichlorophenolindophenol (DCPIP) reductase (complex II)

The assay was performed at 600 nm using as acceptor (80 μM) DCPIP and 10 mM succinate as the donor in a medium containing 10 mM KH2PO4 (pH 7.8), 2 mM EDTA, 1 mg/ml BSA in the presence of 80 μM 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone, 0.24 mM KCN, 4 μM rotenone, 0.2 mM ATP and 0.4 μM antimycin for 2 min. The addition of 10 mM malonate inhibited the oxidation of succinate.

2.2.3. Measurement of ubiquinol cytochrome c reductase (complex III)

The assay was performed at 550 nm using 40 μM oxidized cytochrome c as the acceptor and 80 μM decylubiquinol as the donor in a medium containing 10 mM KH2PO4 (pH 7.8), 1 mg/ml BSA, 2 mM EDTA, in the presence of 0.24 mM KCN, 4 μM rotenone, 0.2 mM ATP for 2 min. The addition of 1 μM antimycin allowed us to distinguish between the reduction of cytochrome c catalyzed by the complex III and the nonenzymatic reduction of cytochrome c by the reduced quinone.

2.2.4. Measurement of the rotenone-sensitive NADH-cytochrome c oxidoreductase (NCCR) (complex I + III)

The assay was performed at 550 nm using 40 μM oxidized cytochrome c as the acceptor and 0.8 mM NADH as the donor in a medium consisting in 10 mM Tris (pH 8.0) containing 1 mg/ml BSA and 0.4 mM KCN for 3 min. The addition of 4 μM rotenone allowed us to quantify the NCCR rotenone-sensitive activity.

2.2.5. Measurement of cytochrome c oxidase (complex IV)

The assay was performed at 550 nm using 10 μM
reduced cytochrome c as donor in a isoosmotic medium (10 mM KH$_2$PO$_4$ (pH 6.5), 1 mg/ml BSA, 0.3 M sucrose) for 2 min, after permeabilizing both mitochondrial membranes with 2.5 mM n-dodecyl-β-D-maltoside [10].

2.2.6. Measurement of citrate synthase (CS)

Performed at 412 nm following the reduction of 2 mM 5,5′-dithio-bis(2-nitrobenzoic acid) in the presence of 0.1 mM acetyl-CoA and 12 mM oxalacetic acid in a medium with 10 mM KH$_2$PO$_4$ (pH 7.8) containing 2 mM EDTA, 1 mg/ml BSA and 0.1% Triton X-100.

2.3. DNA isolation and sequencing of cytochrome b (cyt b) gene

Total DNA was isolated from heart muscle tissue from three IDC patients whose complex III activity was below the normal control range and three controls. Standard procedures based on phenol–chloroform purification and isopropanol precipitation were used [11]. Sequencing of the mitochondrial cyt b gene was performed on polymerase chain reaction (PCR) amplified fragments. A 1378 base pair mtDNA region (nucleotides 14 682 to 16 060) containing the gene coding for cyt b was amplified by PCR by using the primers corresponding to mtDNA positions 14 682–14 709 and 16 060–16 048. DNA fragments were purified from agarose gels by the Qiagen puriﬁcation kit (Qiagen, Chatsworth, CA, USA), and automatically sequenced using 3.5 pmols of sequencing primers and a Taq Dye Deoxy Terminator Sequencing kit (No. 401 113; Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s recommendations. Data analyses were performed with the Collection and Analysis computer programs from Applied Biosystems. Cyt b gene sequences from patients and controls were compared with the Cambridge mtDNA sequence [12].

2.4. Statistics

Qualitative variables are expressed as raw data or percentages, and compared using chi-square tests. Quantitative data are expressed as mean±S.D., and compared by means of analysis of variance (ANOVA) test. When a statistically significant difference was found, a post-hoc Student–Newman–Kreuls test was applied to uncover between which pair of groups the difference was. The relation between quantitative variables was studied by means of linear regression analysis. A $P$-value <$0.05$ was considered significant.

3. Results

3.1. Clinical results

Clinical information of the subjects studied is shown in Table 1. All patients with IDC and IC had NYHA class IV congestive heart failure and were treated with vasodilators, digoxin and diuretics. None of the subjects studied had any current infection or immunological disease. IDC patients had angiographically normal coronary arteries in all cases. There were no other differences in clinical variables or treatment between IDC and IC patients.

3.2. OXPHOS activities

Specific activities of complex I, complex II, complex III, complex IV, complex I+III and CS in IDC, IC patients and controls are presented in Table 2.

Complexes II, III and I+III activities in heart homogenates of both, IDC and IC patients, were significantly lower than those of healthy controls. There were no differences for the rest of enzyme activities.

In order to exclude the possible influence of the number of mitochondria, the enzyme activities of the different complexes were corrected by CS activity (Table 3). While the difference of complex II activity disappeared, the values of complex III activity remained significantly lower in IDC and IC patients with respect to healthy controls, either if it was assessed individually or together with complex I (I+III). Nonetheless, only in three cases of IDC

| Table 1 | Clinical characteristics and left ventricular function data of the patients included in the study$^a$
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<tr>
<td></td>
<td>IDC ($n=17$)</td>
<td>IC ($n=6$)</td>
<td>Controls ($n=17$)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53±7</td>
<td>55±4</td>
<td>59±11</td>
</tr>
<tr>
<td>Male/female</td>
<td>12/5</td>
<td>6/0</td>
<td>12/5</td>
</tr>
<tr>
<td>NYHA IV$^b$</td>
<td>100%</td>
<td>100%</td>
<td>–</td>
</tr>
<tr>
<td>EF$^c$ (%)</td>
<td>20±7</td>
<td>25±3</td>
<td>–</td>
</tr>
<tr>
<td>EDD$^d$ (mm)</td>
<td>75±9</td>
<td>76±4</td>
<td>–</td>
</tr>
<tr>
<td>ESD$^d$ (mm)</td>
<td>64±7</td>
<td>61±8</td>
<td>–</td>
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$^a$ NYHA IV=class IV of the New York Heart Association congestive heart failure classification.

$^b$ EF=Ejection fraction.

$^c$ EDD=End-diastolic diameter.

$^d$ ESD=End-systolic diameter.

| Table 2 | Absolute respiratory chain enzymatic activities and citrate synthase activities in heart homogenates$^a$
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<tr>
<td></td>
<td>IDC ($n=17$)</td>
<td>IC ($n=6$)</td>
<td>Controls ($n=17$)</td>
</tr>
<tr>
<td>Complex I</td>
<td>120±36</td>
<td>133±35</td>
<td>132±61</td>
</tr>
<tr>
<td>Complex II</td>
<td>286±103$^c$</td>
<td>307±115</td>
<td>392±119</td>
</tr>
<tr>
<td>Complex III</td>
<td>371±110$^e$</td>
<td>456±110$^e$</td>
<td>703±266</td>
</tr>
<tr>
<td>Complex IV</td>
<td>578±202</td>
<td>655±393</td>
<td>589±288</td>
</tr>
<tr>
<td>Complex I+III</td>
<td>63±40$^f$</td>
<td>73±22$^f$</td>
<td>163±75</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>422±156</td>
<td>416±106</td>
<td>486±127</td>
</tr>
</tbody>
</table>

$^a$ Activity values expressed as nmol/min/mg prot.

$^b$ ANOVA test for the three groups taken together.

$^c$ These values were significantly lower than control values in the Student–Newman–Kreuls post-hoc test.
To rule out the possibility that a complex III defect could be due to the presence of a mutated form of the single mtDNA coded component of this enzyme, we sequenced the cyt b gene of those three IDC patients showing a decrease in complex III activity and three controls. Two controls and two IDC patients had a T→C change in the position 14 766. A third control had a T→C transition in the position 14 798. All these changes have been previously reported as polymorphisms [13,14].

### 3.3. Sequencing of cyt b

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### 4. Discussion

Impairment of cardiac energy metabolism is considered to be one of the main causes of IDC [15]. Because myocardium depends heavily on oxidative metabolism, mitochondrial dysfunction might have a primary role in such disorders. Actually, cardiac involvement has been found in a great number of mitochondrial diseases, either in association with neuromuscular symptoms or, less frequently, as the main clinical feature [16,17].

In the present study, we assessed respiratory chain enzyme activities in heart homogenate from 17 patients suffering end-stage IDC. The main finding was a significant decrease in enzymatic activity of complex III measured either isolated or together with complex I (I+III), independently if expressed as mg of protein or corrected by CS activity. Marin-Garcia et al. [18] also found an abnormal reduction of several mitochondrial enzyme activities in IDC, complex III being the most frequently decreased. Similar findings had been previously found by other authors [19]. Unfortunately, such studies did not include appropriate control groups. On the other hand, two other studies reported increased respiratory complex III activities in IDC [20,21], suggesting some sort of mitochondrial compensatory mechanism in heart failure. Those later studies, nevertheless, differed from ours in some methodological aspects. The activity of complex III was not measured directly, but through the activity of complexes I+III and II+III. Furthermore, some patients were not in heart failure class IV of the NYHA classification. When we tried to compare the activity of complex III with the classical parameters to measure heart failure, correlations were in general poor, end-diastolic diameter being the nearest to statistical significance. This could reflect the poor correlations that generally exist between classical measures of cardiac function and heart performance in end-stage cardiac failure. Alternatively and in accordance with increased complex III activity reported by others, there may be stages in the evolution of cardiac failure where a compensatory increase in enzyme activities exists, the decrease being a very late event, as found in the present study.

Although the mean complex III enzymatic values in the
IDC group were decreased when compared with noncardiomyopathic controls, only three patients felt below the lower levels of our laboratory normal ranges. It is possible that the rest of IDC patients had only a partial or weak secondary deficiency of this respiratory enzyme. Whatever the case, these three IDC patients were the best candidates for a primary complex III deficiency. When their mtDNA cyt b genome was analysed, we only found modifications from the Cambridge mtDNA sequence [12] previously described in normal controls [13,14]. Thus we have to consider them as neutral polymorphisms and not as mutations with deleterious effects. Accordingly, if this complex III impairment has any genetic basis, we have to assume it is not present in the mtDNA, at least in the form of a cyt b gene mutation. This may indicate that the genetic problem resides in nuclear encoded cyt b subunits or in nuclear genes encoding for mitochondrial translocases [22], components of the protein transport machinery [23] or mtDNA homeostasis [24,25]. However, to our understanding a more likely explanation is that it may simply reflect a secondary phenomenon. Supporting this hypothesis, we also found a decrease in complex III (and I+III) enzymatic activities in patients with IC. The existence of very similar electron transport chain enzyme disturbances in these two types of dilated cardiomyopathies of different aetiology (idiopathic and ischemic) strongly suggests that mitochondrial dysfunction is a secondary event appearing late in a failing heart. These nonspecific electron transport chain abnormalities are in agreement with recent reports demonstrating a reduced state III respiratory rate when glutamate was used as substrate in dogs with experimental IC [26], and decreased oxidative capacity in humans with cardiomyopathies of diverse aetiology [27].

Furthermore, the molecular findings previously reported by other authors in IDC supposedly due to primary mitochondrial defects are extremely variable which would be more in accordance with secondary changes rather than primary defects [2–4]. In a recent study Yücel et al. [28] found increased oxidative stress in erythrocytes and erythrocyte membranes from IDC and IC patients. Additionally Corral-Debrinski et al. [29,30] reported elevated mtDNA deletions and increased amounts of OXPHOS transcripts in chronically ischemic hearts as well as in two patients affected by IDC. It might be that the cardiac dysfunction present in dilated cardiomyopathies produce, either through an increase in free radical production or through other unknown mechanisms, a secondary damage in mtDNA, membranes and proteins, and thus, a decline in some mitochondrial enzyme activities. Similar mechanisms based on a free radical hypothesis have been proposed to explain some neurodegenerative diseases, atherosclerosis and ageing [29,31].

We conclude that the decrease of respiratory chain complex III activity found in both IDC and IC favours the hypothesis of a secondary phenomenon, and not a primary mitochondrial disease in the majority of IDC patients.

Acknowledgements

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References

[14] Howell N, Halvorson S, Burns J, McCullough DA, Poulton J. When chronically ischemic hearts as well as two cases [22], components of the protein transport machinery [23] or mtDNA homeostasis [24,25]. However, to our understanding a more likely explanation is that it may simply reflect a secondary phenomenon. Supporting this hypothesis, we also found a decrease in complex III (and I+III) enzymatic activities in patients with IC. The existence of very similar electron transport chain enzyme disturbances in these two types of dilated cardiomyopathies of different aetiology (idiopathic and ischemic) strongly suggests that mitochondrial dysfunction is a secondary event appearing late in a failing heart. These nonspecific electron transport chain abnormalities are in agreement with recent reports demonstrating a reduced state III respiratory rate when glutamate was used as substrate in dogs with experimental IC [26], and decreased oxidative capacity in humans with cardiomyopathies of diverse aetiology [27].

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