Modification of the functional capacity of sarcoplasmic reticulum membranes in patients suffering from chronic fatigue syndrome

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Abstract

In chronic fatigue syndrome, several reported alterations may be related to specific oxidative modifications in muscle. Since sarcoplasmic reticulum membranes are the basic structures involved in excitation–contraction coupling and the thiol groups of Ca$^{2+}$ channels of SR terminal cisternae are specific targets for reactive oxygen species, it is possible that excitation–contraction coupling is involved in this pathology. We investigated the possibility that abnormalities in this compartment are involved in the pathogenesis of chronic fatigue syndrome and consequently responsible for characteristic fatigue. The data presented here support this hypothesis and indicate that the sarcolemmal conduction system and some aspects of Ca$^{2+}$ transport are negatively influenced in chronic fatigue syndrome. In fact, both deregulation of pump activities (Na$^{+}$/K$^{+}$ and Ca$^{2+}$-ATPase) and alteration in the opening status of ryanodine channels may result from increased membrane fluidity involving sarcoplasmic reticulum membranes.

Keywords: Chronic fatigue syndrome; Membrane fluidity; Ca$^{2+}$ transport; Na$^{+}$/K$^{+}$-ATPase; Ca$^{2+}$-ATPase

1. Introduction

Chronic fatigue syndrome (CFS) is characterized by severe disabling fatigue and specific symptoms, such as musculoskeletal pain, sleep disturbance, impaired concentration, and headaches [1]. Initially CFS was considered to be a disorder associated with psychiatric problems, which has been a significant obstacle in the recognition of CFS as an organic condition. There are two main current definitions: one from the United States Centers for Disease and Prevention (CDC) of 1994 and the second based on Oxford criteria [2,3]. The most important difference between the two criteria is that the American definition includes several physical symptoms secondary to immunological or infective pathologies [4,5], while the British definition focuses on the presence of mental fatigue. CFS affects 0.2–2.6% of the population, depending on the criteria used to define the disease [6]. CFS has similar prevalence in people of different socioeconomic status, affects all ethnic groups, and the only demographic risk factor is gender, since females are more prone to the illness [7,8]. Several morphological and biochemical alterations that may be related to generation of free radicals have been reported [9]. In CFS patients various blood parameters such as malondialdehyde (MDA), methemoglobin, 2,3-diphosphoglycerate levels, and volume of erythrocytes are elevated [10]. Furthermore, the skeletal muscle tissue of CFS patients present a number of abnormalities such as: (a) morphological alterations of myofibrils and fatty/fibrous tissues; (b) inversion of the ratio between cytochrome oxidase and succinate dehydrogenase; (c) pleio/polymorphism and monstruosity of mitochondria; (d) reduction of a number of mitochondrial enzymatic activities and increments in common mitochondrial DNA deletions [11].

We previously demonstrated specific oxidative alterations in the vastus lateralis muscle of CFS patients, both as an increased value of oxidative damage markers (8OH-dG, MDA) and membrane fluidity, as well as imbalances in the oxidant–antioxidant system [12]. These findings are in agreement with the hypothesis that impairment of...
mitochondrial activity underlines an increase in the production of reactive oxygen species (ROS), leading to muscle fatigue, similar to normal ageing [13]. This interpretation is supported by CFS-associated muscle magnesium deficiency, a possible further cause of oxidative stress [14]. An interesting new theory for CFS pathogenesis proposes that excessive cytokine production due to infections is correlated with increased nitric oxide (NO) synthesis [15], thus representing another potential source for oxidative damage [16]. All these data taken together strongly support the hypothesis that oxidative damage plays an important role in the pathogenesis of CFS.

The fatigue that occurs in CFS patients typically fluctuates and is similar to that of multiple sclerosis or chronic inflammatory demyelinating polyneuropathy (CIDP) [17]. Ion channel have been correlated to various pathological states and is similar to that of multiple sclerosis or chronic inflammatory demyelinating polyneuropathy (CIDP) [17]. Also, CFS has been associated with abnormalities of ion channels [18]. It is possible that excitation–contraction (E–C) coupling is involved in this pathology since the thiol groups of Ca²⁺ channels present in the terminal cisternae of the sarcoplasmic reticulum (SR) are specific targets for ROS [19]. In this study, we investigate whether or not the E–C coupling mechanism that takes place in the junctions between sarcoplasmic reticulum and T-tubules is specifically involved in the pathogenesis of CFS.

2. Materials and methods

Our studies were carried out on samples from: (a) four CFS patients, three of whom were males of respectively 30, 33, and 39 years of age, and the other female of 38 years of age; and (b) three patients suffering from fibromyalgic syndrome (FS), one male and two females of respectively 45, 40, and 60 years of age. The latter set of patients was solely analyzed for membrane fluidity. All patients were selected at the CFS Study Center, University of Chieti, on the basis of CDC diagnostic criteria [20]. The control group consisted of six healthy age- and sex-matched subjects who underwent elective orthopedic surgery. Biopsies from the vastus lateralis muscle were taken following a thorough clinical evaluation, which included detailed manual muscle testing. The eventual risks associated with muscle biopsy, which include discomfort during the postoperative period, possible complications of the surgical procedure and expected findings from the biopsy (using informed consent), were explained to the patient.

Biopsy specimens (0.1–0.4 g) were obtained from the vastus lateralis muscle according to the methods of Engel and Franzini-Armstrong [21], and samples were immediately frozen in liquid nitrogen. The following analyses were performed on the biopsy specimens.

2.1. Membrane fluidity

Partially purified SR membranes were prepared from muscle biopsies, as described by Fanò et al. [22]. Protein (100 μg) in 200 μl of 250 mM sucrose was incubated for 1 h in the dark in the presence of 2 mM diphenylhexatriene (2 μl) dissolved in tetrahydrofuran, following the procedure of Shinitzky and Barenholz [23]. Membrane fluidity was measured as fluorescence anisotropy, as described previously [12].

2.2. [3H]PN200-110 binding

Membranes derived from homogenized human skeletal muscle biopsies were prepared according to the procedure of Renganathan et al. [24]. The dihydropyridine receptor (DHPR) concentration was determined using the radioligand [3H]PN200-110. Protein (100 μg) was incubated in a final volume of 250 μl binding buffer in the presence of 1 nM [3H]PN200-110 for 1 h at room temperature, following which samples were filtered with Whatman GF/C filters and washed with 6 volumes of ice-cold washing buffer [24]. Radioactivity was determined by liquid scintillation counting. Non-specific [3H]PN200-110 binding was assessed in the presence of 10 μM unlabeled nifedipine.

2.3. Ryanodine binding

Vesicles derived from the SR were prepared as described by Fanò et al. [22], washed in binding buffer (200 mM KCl, 10 mM HEPES (pH 7.4), 100 μM CaCl₂, 0.1 mM PMSF, 1 μg/ml leupeptin) and centrifuged at 100 000 g for 90 min before resuspension at a final concentration of 1 mg/ml. Aliquots (65 μg) of protein were incubated at 25°C in a final volume of 250 μl in the presence of 10 nM [3H]Ryanodine for 120 min. In another experiment, samples were incubated either with or without 10 mM caffeine, filtered with Whatman GF/C filters and washed with 6 volumes of ice-cold washing buffer [24]. The amount of bound [3H]ryanodine was determined by liquid scintillation counting [25].

2.4. Ca²⁺-ATPase activity

This was measured on 30 μg aliquots of protein in a final volume of 1 ml medium containing 5 mM ATP and 10 μM CaCl₂, similar to a previously reported procedure [26].

2.5. Na⁺/K⁺-ATPase activity

Enzymatic activity was measured on external membranes derived from skeletal muscles, using the modified method of Rock et al. [27]. Samples were homogenized in 20 mM HEPES, 300 mM sucrose, 1% BSA, 500 μM PMSF, pH 7.0 (4 × 20 s) and centrifuged at 8000 × g for 15 min. The supernatant was centrifuged at 40 000 × g for 70 min,
followed by 100 000 × g for 90 min. The pellet was resuspended in 10 mM HEPES, 150 mM KCl, pH 6.8. Protein concentrations were determined by the Lowry method [28]. Protein (25 µg) was incubated in 1 ml medium (134 mM NaCl, 21 mM KCl, 1 mM EGTA, 25 mM HEPES, 2.5 mM ATP, pH 7.5) in the presence or absence of 1 mM ouabain. After 10 min incubation at 37 °C, the reaction was stopped by the addition of 12.5% TCA (1 ml) and the precipitate removed by centrifugation at 5000 × g for 10 min. Released orthophosphate was determined on 1 ml clear supernatant, following the method of Taussky [29].

2.6. Immunoblotting

Immunoblotting was performed on microsomal fractions prepared using the procedure of Carmody et al. [30]. Proteins (50 µg) were resolved on 6% SDS–PAGE for RyR1 detection and transferred to a nitrocellulose membrane using a semi-dry blotting apparatus (500 mA, 3–4 h). Membranes were washed with 30 mM TBS (10 mM Tris–HCl (pH 7.5), 0.9% NaCl) and 6% BSA at room temperature for 1 h. After a further wash with 0.1% TBS/Tween-20, PEG (1 M Tris–HCl (pH 7.5), 200 mM NaCl, 16 mM!ethylene glycol) and 8% FCS, immunoblots were incubated overnight at 4 °C with primary antibody (anti-RyR1, Sigma) solution (1:5000 dilution) in 8% PEG/FCS. Membranes were washed with PEG buffer for 10 min, followed by 0.1% TBS/Tween-20, and incubated with peroxidase-linked anti-mouse antibody diluted in 8% PEG/FCS (1:2000 dilution). Blots were washed with 0.1% TBS/Tween-20. Immunoreactive proteins were detected with enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech) and quantified by densitometry (Imagemaster im1D, Pharmacia Biotech).

2.7. Statistical analyses

Two different tests were used to analyze our data: linear correlation (Pearson’s test) for the membrane fluidity data while the unpaired t-test was used for the binding studies ([3H]PN200-110 and [3H]ryanodine) and Na⁺/K⁺- and Ca²⁺-ATPase activity. Prism-2 from GraphPad Software Inc. (San Diego, CA) was used to automatically perform the statistical analysis.

3. Results and discussion

We previously reported that membrane fluidity increases in both FS and CFS patients in comparison to control subjects [12]. FS patients, who generally exhibit a very similar pathology to CFS, display higher total membrane fluidity than CFS and control samples. Since SR membranes are the basic structures involved in E–C coupling, we examined the possibility that abnormalities within this compartment are responsible for the characteristic fatigue of CFS. Fig. 1 depicts data on membrane fluidity measured in purified SR membranes. Evidently, CFS patients maintain higher fluidity in comparison to controls, whereas FS patients exhibit fluidity values comparable to controls. This was confirmed by functional analyses on the FS muscle Ca²⁺-transport mechanism, which was similar to that of controls (data not shown). Fluorescence polarization values (P) of SR membranes derived from CFS and control muscles exhibit a similar slope, but different absolute values at temperatures between 15 and 40 °C. This finding clearly implies different membrane fluidity in the two experimental groups, suggesting alterations in the pathological samples at the SR level. We hypothesize that SR modifications play a specific role in this disease (as well as in ageing [22]) and may thus be potentially used as a diagnostic marker to distinguish CFS from FS. This modification of membrane fluidity in the pathological samples may correlate to an alteration of polyunsaturated fatty acid, usually target of the lipid peroxidation [31]. We have in fact previously demonstrated an increase of peroxidation in CFS muscles [12]. SR membranes of skeletal fibers usually contain variable, but little, amount of cholesterol [26,32]. Correlation between variability in cholesterol amount and biological functions have never been reported. Because of this, the alterations of membrane properties in CFS could be ascribed to the phospholipids.

The main function of SR is to control cytoplasmic concentration of Ca²⁺. The release of this ion from SR terminal cisternae is controlled by a specific interaction between dihydropyridine receptors (DHPRs), located in the transverse tubule membrane, and the Ca²⁺-release channels of the SR, RyR type 1 in skeletal muscle fibers. It is clear

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**Fig. 1.** Membrane fluidity in human skeletal muscle (vastus lateralis). Fluorescence polarization (P) values of membranes derived from CFS, FS and healthy-matched control subjects at different temperatures (15, 20, 25, 30, 35, 40 °C) are depicted. Also, the statistical analysis of the slope and the intercepts are reported. The values are the means ± SD (n = 3). CFS, chronic fatigue syndrome; FS, fibromyalgic syndrome; C, control.
that any significant alteration of SR membrane structure could affect this interaction and consequently the E–C coupling mechanism. We have tested this possibility measuring the following parameters in both CFS and control samples: (1) the $[^3H]$PN200-110 binding to L-type Ca$^{2+}$-channels as a marker of the amount of DHPRs present in T-tubules membranes; (2) the ryanodine binding to the SR Ca$^{2+}$-release channels as a marker of the ability of SR terminal cisternae to release Ca$^{2+}$; and (3) the activity of Ca$^{2+}$-ATPases as an indicator of the ability of SR membranes to uptake Ca$^{2+}$. As shown in Table 1 (1st row), the small but significant decrease of DHPR binding in CFS samples indicates that this membrane target is also modified by the pathological state (control, 1.4 ± 0.16 vs. CFS, 1.13 ± 0.2 pmol/mg protein; $P < 0.05$). It is additionally important to note that ageing muscle displays decreased DHPR binding [24]. An increased value of Ryanodine binding suggests that SR- Ca$^{2+}$ channels are in an open state, with the consequence that Ca$^{2+}$ release from SR vesicles increases. Table 1 (2nd row) depicts 10 mM $[^3H]$ryanodine binding to SR vesicles derived from the vastus lateralis muscle of CFS patients. The data show that the pathological samples exhibit decreased $[^3H]$ryanodine binding, thereby indicating a diminished capacity of Ca$^{2+}$ channels to be maintained in the open state (controls 0.130 ± 0.016 vs. CFS 0.090 ± 0.005 nmol/mg protein; $P < 0.01$). A possible channel alteration in the muscle of CFS subjects was indirectly confirmed by experiments in which the ryanodine-binding assay was performed in the presence of 10 mM caffeine (an alkaloid that stimulates the opening of SR Ca$^{2+}$ channels) [33]. A further difference between controls and CFS patients is that not only the Rya binding is decreased in the latter, but also the potentiation effect of caffeine on this binding is depressed. In fact, while in controls 10 mM caffeine induces a drastic increase in ryanodine binding (increase of 87 ± 18%), the potentiation of the alkaloid on CFS patients is much smaller (only 49 ± 4%) indicating a possible desensitization of the RyR/Ca$^{2+}$ release channel. The difference between the two data is statistically significant as shown by the $t$-test value; $P < 0.05$. This finding is not particularly surprising and may be the consequence of an alteration of E–C coupling or modification of excitation-Ca$^{2+}$ release mechanism(s). The oxidation status of RyR1 thiols is directly correlated to the functional status of the channel. In fact, oxidation of ca. 10 of these thiols had little effect on channel activity, whereas more extensive oxidation reduced the opening status [34]. In view of the fact that ryanodine binds its receptor in proportion to the opening status, the decrease in binding observed in CFS muscle preparations is probably due to a decreased SR Ca$^{2+}$ channel opening status. On the other hand, this decrease does not depend on a reduction in specific binding sites, since RyR1 expression is not significantly different in CFS samples compared to the controls (Fig. 2).

Table 1 (3rd row) also depicts the activity of the enzyme, Ca$^{2+}$-ATPase type 1 (SERCA 1 [35]), which controls the capacity to recover Ca$^{2+}$ released by terminal cisternae. Vesicles prepared from CFS samples exhibited a significant decrease in sarcoplasmic reticulum Ca$^{2+}$-ATPase activity, compared to controls (3.4 ± 0.38 vs. 2.0 ± 0.05 P, µg/mg/ml per min; $P = 0.0075$). The status of Ca$^{2+}$ release described above and the correlation [36] between enzyme activity and myoplasmic Ca$^{2+}$ concentrations raise the possibility that the decrease in measured activity is a consequence of lower Ca$^{2+}$ availability, secondary to reduced SR Ca$^{2+}$ release.

Na$^+/K^+$-ATPase specific activity is a marker of the physiological capacity of plasmalemma that correlates with both membrane excitability and muscle contractility. The activity of this enzyme (Table 1, 4th row) is increased in the pathological samples (0.83 ± 0.05 vs. 1.69 ± 0.32 P, µg/mg/ml per min; $P < 0.05$) indicating that Na$^+/K^+$ transport is modified in CFS muscle. Higher Na$^+/K^+$-ATPase pumping activity compared to controls may signify an imbalance in sarcosomal Na$^+$/K$^+$ permeability. It is possible that this Na$^+/K^+$ alteration results from the functional modification of Na$^+$ and/or K$^+$ channels as a consequence of alterations in membrane fluidity.

Earlier studies report that oxidative skeletal muscle fibers of spontaneously hypertensive rats exhibit several physiological defects, including a reduced ability to maintain force secondary to increased levels of extracellular K$^+$. This finding correlates with a decrease in Na$^+/K^+$-ATPase

<table>
<thead>
<tr>
<th>Assay</th>
<th>Control</th>
<th>CFS</th>
<th>$P$ value</th>
</tr>
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<tbody>
<tr>
<td>$[^3H]$PN200-110 binding (pmol/mg protein)</td>
<td>1.4 ± 0.16</td>
<td>1.13 ± 0.2</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>$[^3H]$Rya binding (nmol/mg protein)</td>
<td>0.13 ± 0.016</td>
<td>0.09 ± 0.005</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>Ca$^{2+}$/Mg$^{2+}$-ATPase activity (P, µg/mg/ml/min)</td>
<td>3.40 ± 0.38</td>
<td>2.00 ± 0.05</td>
<td>$P = 0.0075$</td>
</tr>
<tr>
<td>Na$^+/K^+$-ATPase activity (P, µg/mg/ml/min)</td>
<td>0.83 ± 0.05</td>
<td>1.69 ± 0.32</td>
<td>$P &lt; 0.05$</td>
</tr>
</tbody>
</table>

First row, specific $[^3H]$PN200-110 binding; 2nd row, specific $[^3H]$ryanodine binding; 3rd row, specific enzymatic Ca$^{2+}$/Mg$^{2+}$-ATPase activity; 4th row, specific enzymatic Na$^+/K^+$-ATPase activity. All assays were performed on homogenized tissue (DHPR binding) and membrane preparations (external for Na$^+/K^+$-ATPase and SR derived for $[^3H]$ryanodine binding and Ca$^{2+}$/Mg$^{2+}$-ATPase) derived from skeletal muscle (vastus lateralis) of CFS patients ($n = 3$) and control ($n = 5$). Experiments were performed in quintuplicate and data are presented as mean ± SEM. $[^3H]$Rya, $[^3H]$ryanodine; CFS, chronic fatigue syndrome; P, inorganic phosphate.
activity, but not with an alteration in the number of sites or the binding affinity of the pump [37]. Interestingly, a decrease in pump site number, but not binding affinity, has been demonstrated in the skeletal muscle of rats with chronic heart failure [38]. In our opinion, upregulation of pump activity may reflect an attempt by the muscle to counteract the effect of dysfunction that is established in this pathology.

4. Conclusions

CFS is a pathological state characterized by alterations of muscle capacity in which the rapid onset of fatigue is associated with muscle pain. Both these symptoms may be due to a modification in membrane organization (mainly the SR network) due to oxidative damage, with consequent possible imbalance in E–C coupling. The data presented here support this hypothesis and indicate that the sarcoplasmic reticulum induces rapid calcium release. In fact, both the deregulation of pump activities (Na⁺/K⁺ transport and mechanical properties in frog skeletal muscle. Free Radic Biol Med 1999;26:303–8.

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