Abstract

Purpose: Since physical exertion is known to exacerbate the symptoms of chronic fatigue syndrome (CFS) and metabolic changes and including oxidative stress can modulate heat shock protein (HSP) expression responses, we sought to determine whether HSP expression is altered in CFS patients before and after exercise. Heat shock proteins (HSPs) in peripheral blood mononuclear cells (PBMC) were examined from 6 chronic fatigue syndrome (CFS) patients and 7 controls before and after a standardized treadmill exercise. Basal hsp27 was significantly higher among CFS patients than in controls (0.54 ± 0.13 vs. 0.19 ± 0.06, mean ± SEM; P < 0.01). In addition, these levels in CFS patients decreased immediately post-exercise (0.25 ± 0.09; P < 0.05) and remained below basal levels at day 1 post-exercises (0.18 ± 0.05; P < 0.05). This declining expression of HSP27 during the post-exercise period among CFS patients was confirmed by one-way ANOVA analysis with repeated measures (P < 0.05). In contrast, HSP27 levels remained relatively constant following exercise among control subjects. Similar patterns of declining

raise the possibility that HSP profiling may provide a more objective biologic marker for this illness.

Methods: HSP27, HSP60, HSP70 and HSP90 expression from 6 CFS patients and 7 age- and sex-matched controls were examined by western blot analysis of peripheral blood mononuclear cells immediately before, after, and at 1 day and 7 days following a standardized treadmill exercise.

Results: Basal HSP27 was higher among CFS patients than in controls (0.54 ± 0.13 vs. 0.19 ± 0.06, mean ± SEM; P < 0.01). In addition, these levels in CFS patients decreased immediately post-exercise (0.25 ± 0.09; P < 0.05) and remained below basal levels at day 1 post-exercises (0.18 ± 0.05; P < 0.05). This declining expression of HSP27 during the post-exercise period among CFS patients was confirmed by one-way ANOVA analysis with repeated measures (P < 0.05). In contrast, HSP27 levels remained relatively constant following exercise among control subjects. Similar patterns of declining
HSP levels in CFS patients were also observed for HSP60 (0.94 ± 0.40 vs. 1.32 ± 0.46; \( P < 0.05 \)), and for HSP90 (0.34 ± 0.09 vs. 0.49 ± 0.10; \( P < 0.05 \)) at day 7 post-exercise compared with basal levels, respectively. In contrast, HSP60 levels in control subjects increased at day 1 (1.09 ± 0.27) and day 7 (1.24 ± 0.50) post-exercise compared to corresponding levels immediately post-exercise (0.55 ± 0.06) (\( P < 0.05 \), respectively).

**Conclusion:** These preliminary findings suggest an abnormal or defective adaptive response to oxidative stress in CFS, and raise the possibility that HSP profiling may provide a more objective biologic marker for this illness.

Chronic Fatigue Syndrome (CFS) is characterized by a constellation of symptoms including debilitating fatigue, myalgia, subjective cognitive and sleep impairment, headaches, and frequently depression.\(^1\) These symptoms are often exacerbated by physical exertion. The etiological factors and pathogenesis remain elusive, despite numerous investigations into various causes including viral infections\(^2\), immunologic abnormalities \(^3\), disorders of muscle mitochondria \(^4\), and metabolic derangements \(^5\).

The heat shock response is a universal and essential adaptive mechanism which permits cells to respond to a broad variety of otherwise detrimental conditions.\(^6\) Under normal physiologic conditions, heat shock proteins (s) are expressed constitutively at basal levels and perform pivotal roles in protein folding and translocation across membranes. When induced under stress, including changes in temperature, glucose depletion, oxidative stress, virus infection and other pathological conditions, HSP’s confer a cytoprotective state through numerous cellular and metabolic responses.\(^7\)

HSPs are classified into different families based upon their apparent molecular mass (small HSP’s, HSP60, HSP70, and HSP90). Small HSP’s, such as HSP27, are important in microfilament organization, cell growth and differentiation, and in protecting cells against apoptosis induced by hyperthermia, inflammatory cytokines and oxidative stress.\(^8\) High expression levels of HSP27 in tumours have been shown to aggravate pathological conditions by mediating a protective effect against apoptosis and by interfering with the activities of chemotherapeutic agents.\(^9\) HSP60 and HSP70 are involved in the oligomeric assembly and transport of peptides associated with the mitochondrial matrix.\(^6\) In addition to heat shock responses, HSP70 also mediates cyto-protection to oxidative stress.\(^10\) HSP90 is one of the most abundant chaperones in the eukaryotic cytosol, and plays a critical role in regulating cellular processes such as hormone signalling and cell cycle control.\(^11\) Importantly, physical exertion and endurance training in healthy athletes have been shown to elevate protective heat shock proteins and antioxidant enzymes in peripheral blood leukocytes.\(^12;13\) Since physical exertion is known to exacerbate the symptoms of CFS \(^14\) and metabolic changes including oxidative stress can modulate HSP expression \(^12\), we sought to determine if differential HSP expression in members of each of the four HSP families could be detected before and after strenuous exercise in the peripheral blood mononuclear cells (PBMCs) of CFS patients compared with age- and sex-matched healthy controls. Our working hypothesis was that CFS patients may display an abnormal or defective HSP response following strenuous exercise.

**Materials and methods**

**Study populations and exercise conditions**

Six CFS patients (1 male, 5 female; mean age ± SD, 51.5 ± 8.46) who fulfilled the diagnostic criteria of the US Center for Disease Control \(^1\) were enrolled during September 2000 and July 2001. Seven healthy volunteers (2 male, 5 female; mean age ± SD, 52.3 ± 6.40) served as age- and sex-matched controls. All provided written consent in accordance with the requirements of the University of British Columbia Clinical Ethics Committee. To control for confounding illness and physical activity, CFS patients also met the following inclusion criteria: a) illness duration < 10 years; b) not taking antidepressants; and c) no past or present his-
tory of bipolar disorder, melancholic depression, and/or psychosis. Control subjects were recruited from a volunteer pool with the following selection criteria: matching age (± 1 year) and sex with an enrolled CFS patient, no known underlying illness, and a sedentary lifestyle that did not involve regular exercise or strenuous activity at work.

All subjects followed a treadmill exercise according to a modified Bruce Protocol originally designed for post-myocardial infarction patients whose history suggested the onset of symptoms at a low workload. The exercise routine consisted of 6 segments of 3 minutes each on the treadmill at a speed of 1.7 m.p.h. and 2.5 m.p.h., respectively, each with the grade set at 0, 5 and 10 degrees consecutively. All subjects were asked to exercise for 18 minutes or until they felt too fatigued to continue.

Blood was collected immediately before and after, and at 1 and 7 days following the exercise tests. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation over Histopaque 1.077 (Pharmacia Fine Chemicals, Dorval, Quebec, Canada) as described previously, and were stored at -20°C until ready for testing.

Cell lysis and detection of HSP's by Western blotting

HSP27, HSP60, HSP70 and HSP90 in the PBMC of CFS patients and control subjects before and after exercise were detected in cell lysates by immunoblot analysis. Samples were rapidly thawed and aliquots were treated on ice for 40 min with a detergent lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 200 μg/mL phenylmethyl-sulfonyl fluoride (PMSF), 2.0 μg/mL aprotinin, 2% Triton X-100 and 1.15 mM PMSF in isopropanol. Cell debris was pelleted at 12,000 g for 4 min, and the total protein concentration in the supernatant was determined by the Bradford assay according to manufacturer’s instructions (Pierce Biotechnology Inc., Rockford, IL).

Test samples from CFS patients and control subjects (50 μg per sample) along with various standards including human recombinant HSP27 (SPP-715), HSP60 (NSP-540), HSP70 (SPP-755), HSP90 (SPP-770) (25 ng each, all from Stressgen Biotechnology Corp., Victoria, BC) and actin (50 ng, Sigma Chemical Co., St. Louis, Mo.) were electrophoresed separately in sodium dodecyl sulphate (0.1%) polyacrylamide gels (12% for HSP27, 10% for HSP60 and HSP70, 8% for HSP90) under reducing conditions in Tris-glycine buffer (pH 8.6). After electrophoresis, proteins were transferred to nitrocellulose membranes (IPV H00010, Millipore Corp., Billerica, Mass.) using a horizontal transblot apparatus (BioRad Laboratories, Hercules, CA). After blocking non-specific reactivity for one hour in 3% skim milk, the membranes were incubated overnight at 4°C on a shaking platform with the following primary mouse monoclonal antibodies (1:1000 dilution in 10 mL 3% skim milk blocking solution): anti-HSP27 (Stressgen SPA-800), anti-HSP60 (Stressgen SPA-806), anti-HSP70 (Stressgen SPA-810B), anti-HSP90 (Stressgen SPA-830), and anti-actin (Sigma A4700). Membranes were washed three times for 10 min, each with Tris-buffered saline containing 0.05% Tween 20 (TBS-T), and incubated for 1 h at room temperature with a goat anti-mouse IgG secondary antibody conjugated with horse radish peroxidase (BioRad). Following three washes with TBS-T, the membranes were incubated with chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, PIERCE) for 5 min and developed after a 2-minute exposure to chemiluminescence-sensitive film (Hyperfilm ECL, Amersham Biosciences, Piscataway, NJ). To minimize possible bias, all test samples were first coded and sequential specimens from the same patient or control subject were tested in the same experiment without knowledge of the source of the specimens.
Western blot images were scanned electronically using the Epson Expression 1600 scanner, and densitometric analysis of the HSP and actin bands was performed using the TN Image Software version 3.2.17 (http://brneurosci.org/tnimage.html). Density units for each HSP were obtained after subtracting background levels and normalizing for actin levels concurrently determined in the same sample. Statistical analyses were performed using the GraphPad Prism version 4.0 software (GraphPad Software, Inc., San Diego, CA). Unpaired and paired Student t-tests (one-tail) were used to assess the significance of differences between CFS patients and control subjects, and between paired samples from the same individual at different time intervals, respectively. To assess the temporal relationship in HSP expression during the post-exercise period, analysis by one-way ANOVA with repeated measures was performed for each group. Differences were considered significant if the probability of the null hypothesis was less than five percent ($P < 0.05$).

**Results**

Representative Western blots demonstrating the detection of HSP27, HSP60, HSP70 and HSP90 in the PBMC lysates obtained from CFS patients and control subjects immediately before (Pre) and after (Post-0), and at 1 day (Post-1) and 7 days (Post-7) following exercise, are shown in Figure 1. Actin levels (~43 kDa) in the same sample were determined concurrently by densitometry and were used to calculate the normalized HSP expression levels for statistical analysis (see details under Materials and Methods). Immunoreactive breakdown products were observed for HSP70 and HSP90, but not for HSP27 or HSP60. Densitometric values were obtained from the bands corresponding to the recombinant HSP preparations but not the breakdown products. Grouped and actin-normalized HSP densitometric units (mean ± SEM) from CFS patients and control subjects before and after treadmill exercise are shown in Figure 2.
Before exercise, HSP27 levels in CFS patients were higher than those in control subjects (0.54 ± 0.13 vs. 0.19 ± 0.06; \( P < 0.01 \), unpaired t test) (Figure 2, panel A). Furthermore, HSP27 levels in CFS patients decreased immediately following exercise (0.25 ± 0.09; \( P < 0.05 \), paired t test) and remained lower at one day post-exercise (0.18 ± 0.05; \( P < 0.05 \)). To further examine the temporal effect on HSP27 expression during the post-exercise period, an analysis by one-way ANOVA with repeated measures was performed. This analysis confirmed a reduction in HSP27 levels post-exercise among CFS patients (\( P < 0.05 \)), but not among control subjects. In contrast, HSP27 levels in control subjects remained relatively constant before and after the standardized exercise tests.

Basal HSP60 levels (Figure 2, panel B) in CFS patients were not higher than those in control subjects before exercise (1.32 ± 0.46 vs. 0.62 ± 0.13; \( P: \text{NS} \)). However, immediately post-exercise, HSP60 levels became higher than control subjects (1.42 ± 0.40 vs. 0.55 ± 0.06; \( P < 0.05 \)). Furthermore, whereas HSP60 levels in control subjects either remained relatively constant before and following exercise (HSP27, HSP90), or increased during the post-exercise period (HSP60).
immediately post-exercise levels (1.42 ± 0.40; \( P < 0.05 \)), respectively.

No differences in HSP70 levels (Figure 2, panel C) were observed between CFS patients and control subjects either before or after exercise. For HSP90 (Figure 2, panel D), a decline from pre-exercise levels was observed in CFS patients at day 7 (0.34 ± 0.09 vs. 0.49 ± 0.10; \( P < 0.05 \), paired t test). In contrast, HSP90 levels remained relatively constant before and following exercise among control subjects.

**Discussion**

Chronic fatigue syndrome is an illness characterized by disabling fatigue of at least six months duration, and is accompanied by a constellation of rheumatologic, musculoskeletal and neuropsychiatric symptoms that are often exaggerated by physical exertion. The pathogenesis of CFS remains unclear but there appears to be a strong link between CFS and infection, inflammation, and immunological or neurological disease and function.17 Additionally, several studies have demonstrated changes in post-exercise physiological events in CFS patients compared with healthy subjects.18 In particular, an enhanced activity of oxidative stress has been demonstrated in resting CFS patients and post-exercise.18-20 Thus, impairment of mitochondrial oxidative phosphorylation and antioxidant defences have been implicated in the pathogenesis of CFS.18,21

In the current study, differences were observed in the expression of HSP27 by CFS patients compared with control subjects, both in basal expression and following strenuous exercise. Basal expression of HSP27 was higher among CFS patients, and its expression declined during the post-exercise period. In contrast, HSP27 in control subjects remained relatively constant before and following exercise. The reduction in HSP27 levels during the post-exercise period among CFS patients was confirmed by a one-way ANOVA analysis with repeated measures (\( P < 0.05 \)). A similar trend in decline of HSP60 and HSP90 levels following exercise was also observed in CFS patients. In contrast, HSP60 levels in control subjects increased during the post-exercise period (\( P < 0.05 \)). No other differences before or after exercise were observed for HSP70 either for CFS patients or control subjects.

This is the first report of HSP expression profiles before and after strenuous exercise in CFS patients compared with healthy control subjects using a standardized exercise protocol. Regular endurance training in healthy athletes has been shown to modulate different HSPs following exercise, particularly those that are implicated in cyto-protective and anti-oxidant function. For example, Fehrenbach et al 12 reported that mRNA levels for both HSP27 and HSP70 in peripheral blood leukocytes increased immediately following acute exertion, and that the corresponding HSPs remained elevated until 24 h post-exercise. Furthermore, basal expression of these HSPs was lower among trained athletes than in untrained control subjects. Thus, upregulation of HSP levels may be an important adaptive physiological response that provides an anti-oxidant and cyto-protective role in skeletal muscle and other tissues following physical exertion.22,23 CFS patients are less able to respond to oxidative stress, such as during strenuous exercise, compared with healthy control subjects.21 Thus, it is of interest that our exploratory study revealed a general trend of higher basal levels HSP27 in PBMC lysates among CFS patients, and that both HSP27, HSP60 and HSP90 declined following treadmill exercise in contrast to control subjects.

The importance of our findings remains to be determined. However, they suggest an underlying mal-adaptation in response to oxidative and other forms of stress among patients with CFS. HSP27 and other small HSPs are over-expressed under oxidative stress and heat shock conditions, forming large oligomers that function to protect the cells from increasing accumulation of oxidized proteins.24 When induced under conditions of stress, HSP27 also serves to prevent apoptosis and the destruction of different anti-oxidant enzymes. Moreover, the upregulation of HSP27 in-
creases the activity of enzymes that maintain intracellular redox conditions (i.e. glucose-6-phosphate dehydrogenase, glutathione reductase and glutathione transferase). Thus, HSP27 appears to mediate cellular anti-oxidant protection by reducing the number of reactive oxygen species and by abating the toxicity of oxidized proteins, the latter mechanism possibly via the ubiquitin-independent 20S proteasome pathway. Expression of HSP27 and other small HSPs has also been correlated to decreases in intracellular iron levels which would otherwise catalyze the formation of hydroxyl radicals and generate oxidized proteins. Conversely, the constitutive over-expression of HSP27 has been shown to result in the enhanced susceptibility of cells to oxidative stress, suggesting that a careful balance of HSP27 expression must be maintained.

Our intriguing findings that PBMC levels of HSP27, HSP60 and HSP90 among CFS patients declined following strenuous exercise in contrast to age- and sex-matched sedentary control subjects suggests a defective adaptive response to oxidative stress among CFS patients. The higher basal expression of HSP27 among CFS patients is also in keeping with the notion that cells from CFS patients are more susceptible to oxidative stress. Collectively, these preliminary observations raise the possibility that CFS patients may lack an efficient adaptive or coping mechanism in response to oxidative stress following strenuous exercise. This is supported by previous reports of increased circulating oxidative stress coupled with diminished antioxidant defences in CFS patients compared to healthy individuals.

Our exploratory study has several limitations. The number of patients and controls in this preliminary study was small. The exercise protocol, although standardized and considered to be strenuous by CSF patients, may be perceived as an insufficient stressor for the control group, as evidenced by minimal changes in their HSP levels post-exercise. However, this exercise regimen was chosen in order to facilitate completion by both the study and control groups, since a more rigorous exercise regimen would not have been tolerated by CFS patients. Nevertheless, despite the possibility that this exercise regimen might have masked an appropriate adaptive response to physical exertion in the control subjects, significant differences in HSP expression between CSF patients and control subjects were still observed. Also, Western blot analysis is an insensitive method for the detection of HSPs in PBMC. Intracellular or surface staining for HSP's followed by FACS analysis may be a more sensitive or accurate approach. The presence of degradation products in the Western blots may also be a confounding variable. These degradation products could have been the result of prolonged storage and repeated freezing and thawing of the test samples, since protease inhibitors were not added to the lysates during storage. Finally, since both HSP60 and HSP70 have been linked to inflammatory processes, it would have been of interest to characterize the expression profiles of inflammatory cytokines in the PBMC of these patients as well as their HSPs. Nevertheless, despite these limitations, our study revealed a consistent trend in HSP expression profiles among CFS patients that differed from age- and sex-matched healthy controls both before and following strenuous exercise. These intriguing findings are also consistent with a growing literature that suggests abnormalities in energy metabolism and capacity to deal with oxidative stress in CFS. These findings not only provide additional avenues for investigating the cellular and metabolic adaptive responses in CFS, but also may offer a more objective biologic marker for identifying patients with this illness.

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