Acute Psychosocial Stress-Mediated Changes in the Expression and Methylation of Perforin in Chronic Fatigue Syndrome

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Abstract: Perforin (PRF1) is essential for immune surveillance and studies report decreased perforin in chronic fatigue syndrome (CFS), an illness potentially associated with stress and/or infection. We hypothesize that stress can influence regulation of PRF1 expression, and that this regulation will differ between CFS and non-fatigued (NF) controls. We used the Trier Social Stress Test (TSST) as a standardized acute psychosocial stress, and evaluated its effect on PRF1 expression and methylation in CFS (n = 34) compared with NF (n = 47) participants. During the TSST, natural killer (NK) cells increased significantly in both CFS (P = <0.0001) and NF subjects (P = <0.0001). Unlike previous reports, there was no significant difference in PRF1 expression at baseline or during TSST between CFS and NF. However, whole blood PRF1 expression increased 1.6 fold during the TSST in both CFS (P = 0.0003) and NF (P = <0.0001). Further, the peak response immediately following the TSST was lower in CFS compared with NF (P = 0.04). In addition, at 1.5 hours post TSST, PRF1 expression was elevated in CFS compared with NF (whole blood, P = 0.06; PBMC, P = 0.02). Methylation of seven CpG sites in the methylation sensitive region of the PRF1 promoter ranged from 38%-79% with no significant differences between CFS and NF. Although, the average baseline methylation of all seven CpG sites did not differ between CFS and NF groups, it showed a significant negative correlation with PRF1 expression at all TSST time points in both CFS (r = −0.56, P = <0.0001) and NF (r = −0.38, P = <0.0001). Among participants with high average methylation (≥65%), PRF1 expression was significantly lower in CFS than NF subjects immediately following TSST. These findings suggest methylation could be an important epigenetic determinant of inter-individual differences in PRF1 expression and that the differences in PRF1 expression and methylation between CFS and NF in the acute stress response require further investigation.

Keywords: pore forming cytotoxic proteins/genetics, transcription, genetic/immunology, DNA methylation, biological stress, fatigue syndrome, chronic/blood
**Introduction**

Stress, whether physical or psychosocial, results in the activation of the hypothalamic pituitary adrenal (HPA) axis and the secretion of cortisol. Studies of chronic fatigue syndrome (CFS) have identified a decreased cortisol awakening response resulting from glucocorticoid-resistance, hypersensitivity to the negative feedback action of cortisol, or altered serotonergic activation of the hypothalamus. In addition to these HPA axis findings, alterations in immune function have been documented in CFS. These include a shift from Th1- to Th2-type T-cell responses, and reduced cytotoxic activity of both natural killer (NK) and CD8+ cytotoxic T-cells. Stress can influence the immune system through its interactions with the HPA axis and the sympathetic nervous system (SNS). Increased SNS activity has been indicated in CFS, and could influence reduced cytotoxicity by suppressing Th1 and enhancing Th2 T-cell responses, resulting in reduced cytotoxic activity in humans and mice. Perforin is a pore-forming protein that acts with granzymes and caspases to induce apoptosis in targeted cells, and reduced perforin expression or function results in reduced cytotoxicity of NK and CD8+ T-cells. Since perforin is important for the cytolytic activity of both NK and cytotoxic CD8+ T-cells, stress regulation of the immune system may be in part mediated through perforin. Studies from three different groups identified reduced perforin in CFS subjects. An 8-fold decrease in perforin (PRF1) messenger ribonucleic acid (mRNA) in peripheral blood mononuclear cells (PBMC) was identified by differential display Polymerase Chain Reaction (PCR). Reduced function or expression of PRF1 in CFS patients has been shown repeatedly; generally, reduced intracellular perforin protein is correlated with PRF1 mRNA down regulation, although one study found reduced cytotoxicity occurred with increased PRF1 mRNA expression. Studies indicate that methylation plays a role in the regulation of PRF1 expression. Hypomethylation of the PRF1 promoter, particularly in the 452 bp methylation-sensitive region (MSR), has been associated with increased expression of PRF1. In vitro reporter gene assays showed that 70% of total PRF1 mRNA expression could be reduced by methylation of the 7 CpG sites in the MSR. In addition, overnight treatment of T-cells with a methylation inhibitor, 5-azacytidine, led to increased PRF1 mRNA expression.

Stress regulation of perforin has not been directly explored. We hypothesize that stress can influence regulation of PRF1 expression, and that this regulation will differ between CFS and non-fatigued (NF) controls. We used the Trier Social Stress Test (TSST), an established and validated method to study the stress response by activating the sympathetic nervous system and the HPA axis, as a standardized acute psychosocial stress, and evaluated its effect on PRF1 expression and methylation in CFS (n = 34) compared with NF (n = 47) participants.

**Materials and Methods**

**Subjects**

The Centers for Disease Control and Prevention (CDC) Human Subjects committee approved the study protocol, which met the ethical standards of the Helsinki Declaration, and all subjects gave written informed consent. This study included the 81 subjects (34 CFS and 47 NF) from a population-based follow-up study of CFS in Georgia, USA who had completed the TSST as part of a three-day study in the Emory General Clinical Research Center. CFS cases met the 1994 international research definition of CFS as evaluated by standardized questionnaires, including the Multidimensional Fatigue Inventory, the SF-36® Health Survey, and the CDC Symptom Inventory. There were no statistical differences between CFS and NF groups in the mean age (CFS, 45 ± 9 years; NF, 46 ± 9 years; P = 0.75), sex (CFS, 82% female; NF, 77% female; P = 0.53), race (CFS, 76% Caucasians; NF, 85% Caucasians; P = 0.23) or body mass index (CFS, 28 ± 5; NF, 26 ± 5; P = 0.075).

**Trier social stress test**

The TSST was performed to assess stress-induced regulation of neuroendocrine, autonomic and immune responses to challenge. The TSST was consistently started at the same time of day to ensure a similar diurnal response between subjects. The test consists of a preparatory and anticipation phase (beginning at 1:15 pm) and a subsequent 10-minute public speaking and 10-minute mental arithmetic task in front of three trained staff members (TSST panel, 1:30 pm to 1:50 pm). An indwelling catheter was placed at 7:30 am for blood draws. Blood was collected in Tempus™ tubes (Applied Biosystems, CA) for microarray analysis at 8:00 am and at 1:00 pm baseline, as well as immediately prior to the TSST panel at 1:30 pm, immediately...
following the TSST panel at 1:50 pm, and at subsequent fifteen minute intervals until 3:05 pm. Blood was also collected in 8 mL Cell Preparation Tubes (CPT™) for PBMC isolation (BD Biosciences, CA) at 10:00 am (3.5 hours prior to TSST) and at 3:05 pm (1.5 hours post TSST). Cell counts were determined in blood collected in Ethylenediaminetetraacetic acid (EDTA) tubes corresponding to all Tempus™ tube blood collection times except 8:00 am.

**Blood processing and DNA/RNA extraction**

Blood drawn into CPT™ was processed within 1.5–5 hours to isolate PBMCs according to the manufacturer’s instructions. PBMCs were frozen in RPMI 1640 media (Invitrogen, CA) at 5 × 10⁶ cells/mL, and stored in liquid nitrogen until use. Deoxyribonucleic acid (DNA) and RNA were isolated from aliquots of the stored PBMC. PBMC RNA was isolated using Trizol (Sigma Aldrich, MO) and PBMC DNA was extracted using the Roche DNA Isolation Kit for Mammalian Blood (Roche Applied Science, IN) following the manufacturer’s protocol.

Tempus™ tubes were frozen at −20°C until extraction (<one month). Whole blood RNA was extracted from Tempus™ tube blood using the 5 PRIME Perfect Pure RNA Cultured Cell Kit (Fisher Scientific, PA). For all samples, RNA quality and quantity were assessed using Agilent 2100 Bioanalyzer RNA Nano Chips (Agilent Technologies, CA) and a Nanodrop 1000 spectrophotometer (Thermo Scientific, DE).

EDTA tubes were submitted to Quest Diagnostics (Atlanta, GA) on the day of collection for determination of Complete Blood Count with differential and flow cytometric determination of T, B, and NK cell counts and percentages.

**Microarray procedure**

Microarray analysis was carried out as previously described,²⁵ using whole blood RNA. One microgram of RNA was labeled using the Exon WT Sense Target Labeling Assay (Affymetrix, CA) and after hybridization to the Affymetrix Human Exon 1.0 ST array, chips were scanned using the Affymetrix GeneChip Scanner 3000. Array analysis was performed using Affymetrix® Expression Console™ (v 1.1) at the transcript level using core-level probe sets. For this analysis, only PRF1 expression was used from this microarray data set.

**Quantitative reverse transcription PCR (qRT-PCR)**

PBMC RNA (500 ng) was DNase I treated in a 10 µL volume using the MessageClean® Kit (GenHunter, TN) and then reverse transcribed in the same tubes using 20 µL reactions with Superscript™ III (Invitrogen, CA) and a combination of oligo(dT) and random hexanucleotide primers (2.5 µM each). LightCycler PCR (20 µL) was performed using the SybrGreen 480 Master Mix (Roche Applied Sciences) that contained 2 µL of 1:20 dilution of complementary DNA (cDNA) and 0.5 µM of each primer. Thermal cycling conditions were as follows: 1 cycle of 94°C for 5 minutes (min), 50 cycles of 94°C 15 seconds (s), 62°C 15 s, and 72°C 15 s. All reactions were carried out in duplicate with previously described peptidylprolyl isomerase B (PPIB),²⁶ and PRF1 primers (forward 5’AGG AGC TGG GCA GAA GGA CAA GA 3’, reverse 5’CAC CAT AGA GGG CTC AAG GGA 3’, product 88 bp).³ PCR efficiencies of PRF1 and PPIB reactions were 1.96 and 1.97 respectively. Relative quantitation was done using the 2⁻ΔΔCT method using the equation 2⁻((sample PRF1 Ct – PPIB Ct)-(calibrator PRF1 Ct – PPIB Ct)) where the calibrator was a 1:100 dilution of HeLa cell cDNA (prepared as above for PBMC cDNA), included in each plate.

**Quantitative methylation by bisulfite-pyrosequencing**

PBMC DNA (200 ng/reaction) was bisulfite treated using the Epitect Bisulfite Kit (Qiagen, CA) according to the manufacturer’s instructions. Bisulfite-pyrosequencing was conducted as previously described to examine methylation levels at seven CpG sites in the MSR of the PRF1 promoter (Fig. 1; sites –876, –776, –744, –720, –691, –670 and –650 base pairs upstream of the transcription start site).²⁹ Three amplicons, D, E and F, and a total of five sequencing primers were used to cover all seven CpG sites. We used a touchdown PCR that consisted of one cycle of 94°C for 5 min for the initial denaturation step followed by 5 cycles each of denaturation at 94°C for 30 s. This process involved varying annealing temperatures for 30 s, and an extension at 72°C for 30 s. Annealing temperatures for the touchdown portion were as follows: 65°C for 5 cycles, 62°C for 5 cycles, 59°C for 5 cycles, 56°C for 5 cycles and 52°C for 5 cycles. And a further 25 cycles of the following: 94°C for 30 s, 50°C for 30 s and 72°C for 30 s. PCR was terminated after a final cycle at 72°C.
for 7 min. The PyroGold Kit was used in conjunction with the PSQ 96MA instrument (Qiagen), and each pyrosequencing reaction used 20 µL of PCR product. All reactions were carried out in duplicate.

Bioinformatic analysis of the PRF1 MSR
Bioinformatic analysis of transcription factor binding sites (TFBS) in the MSR was carried out using the Genomatix Matinspector database (Genomatix Software GmbH (http://www.genomatix.de)). The identified TFBS are as follows: HIF-1 ancillary sequence family, previously identified as AP-2 (HAS/AP-2), E-Twenty Six family (ETS), E2A basic helix loop helix family (E2A), Glucocorticoid Response Element (GRE), Signal Transduction and Activator of Transcription (STAT), and p53 transcription factor family (P53; Fig. 1).

Statistical analysis
Statistical analysis was carried out using either SAS version 9.3 or SAS enterprise guide version 4.3. Normality was tested using the D’Agostino and Pearson Omnibus Normality Test, and parametric tests including two-tailed t-tests and Pearson correlation coefficients were used for analysis.

Results
Impact of TSST on blood cell counts and PRF1 expression
As shown in Figure 2A, there was a significant increase in the overall percentage of NK cells in both CFS \( (P < 0.0001) \) and NF \( (P < 0.0001) \) subjects during the TSST, with no significant difference between groups. Similar to NK cells, other cell type percentages (neutrophils, T-cells, and B-cells) also increased in response to TSST and did not differ with respect to disease status (data not shown).

Microarray analysis of whole blood (Fig. 2B) showed a significant increase in PRF1 expression between the introduction/preparation phase and immediately prior to the TSST oral presentation \((1:00 \text{ pm} - 1:30 \text{ pm})\) of about 1.6-fold in both NF \( (P < 0.0001) \) and CFS \( (P = 0.0003) \). PRF1 expression continued to increase in NF subjects during the TSST oral presentation \((1:30 - 1:50 \text{ pm})\) whereas expression decreased slightly in CFS subjects, resulting in a significant difference between NF and CFS subjects \( (P = 0.04) \) at 1:50 pm. Over the 45 minutes following the TSST, PRF1 expression declined and reached baseline levels in both groups (sampling time 2:35 pm). PRF1 expression in NF subjects continued to decrease, but increased in CFS subjects, resulting in 1.3-fold higher expression in CFS than NF at 3:05 pm, \( P = 0.06 \) (Fig. 2B). PRF1 expression at 10:00 am and 3:05 pm in PBMCs determined by qRT-PCR (Fig. 2C) were generally consistent with whole blood microarray results. PRF1 expression in PBMCs did not differ between CFS and NF at 10:00 am, but at 3:05 pm, PRF1 expression was higher in CFS than NF (1.4 fold, \( P = 0.02 \)).

Impact of TSST on PRF1 methylation
Site-specific CpG methylation in the MSR at 10 am ranged from 38% to 79% and increased at all 7 CpG sites by 2.1% to 4% after the TSST (at 3:05 pm) for the
study population as a whole ($P < 0.0001$ to $P = 0.01$, Fig. 3A). However, in CFS subjects (Fig. 3B) the increase in methylation levels after TSST were significant at only two CpG sites ($−776$ and $−744$) whereas in NF controls (Fig. 3C) the increase was significant at four CpG sites ($−876$, $−744$, $−691$, and $−670$). There were no significant overall differences between CFS and NF in terms of site-specific methylation or average methylation of all 7 CpG sites before or after the TSST.

**Impact of PRF1 methylation on its expression**

To examine the impact of PRF1 methylation on PRF1 expression, we examined correlations between average methylation of the 7 CpG sites and gene expression for each participant and time point in whole blood and PBMCs. Average baseline methylation (10:00 am) was negatively correlated with PRF1 expression in whole blood at all 8 time points (Table 1) in both CFS and NF, statistically significant for all except one NF time point (2:35 pm). Average methylation at 3:05 pm was also negatively correlated with whole blood PRF1 expression at 3:05 pm (Table 1) in both NF and CFS subjects, although correlation was not significant in CFS. Similar negative correlations between PRF1 methylation and PRF1 expression in PBMCs were observed in both NF and CFS subjects (Table 1).

We used linear regression to quantify the relationship between PRF1 average methylation at 10 am and PRF1 expression in PBMCs.
and whole blood PRF1 expression at all time-points (Fig. 4). In CFS subjects, this analysis predicted a 3% increase in PRF1 expression (95% confidence interval 2.5% to 3.7%) with every 1% decrease in PRF1 methylation (y = -0.044x + 12; the antilog of 0.044 = 1.03). A similar inverse relationship was found with NF subjects, however 31% and 14% of the variance in PRF1 expression was explained by PRF1 promoter methylation in CFS (R² = 0.3133) and NF (R² = 0.1425) respectively.

We used the median split of the average methylation level of the 7 CpG sites at 10:00 am to categorize subjects into high (>65%) and low (<65%) methylation. Whole blood PRF1 expression was significantly higher in the low methylation group at all TSST time-points (P-value from 0.0002 to 0.05; Fig. 5A). When the high or low methylation group was stratified by illness, a significant difference was noted between CFS and NF only at the 1:50 pm time-point (immediately following TSST). The CFS high-methylation group had a significantly lower PRF1 expression than the NF high-methylation group (P = 0.005, Fig. 5B).

**Discussion**

This study demonstrates that acute psychosocial stress impacts PRF1 gene expression, and that PRF1 methylation contributes to individual differences in PRF1 expression. PRF1 expression increased approximately 1.6-fold over baseline in response to the TSST and this level of change falls within the relative expression change in perforin seen in other paradigms.33–35 However, the peak PRF1 expression in response to TSST was reduced in CFS compared to NF subjects (Fig. 2B, P = 0.04). NK cells increased in response to the TSST in both CFS and NF, and since PRF1 expression is mostly restricted to NK cells,36 it is likely that the increased expression of PRF1 as a response to the TSST could be related to NK cell numbers. Since we
did not delineate the developmental heterogeneity in NK cells, we cannot distinguish whether all NK cells or only a proportion of mature NK cells contributed to \textit{PRF1} expression following TSST. Further analyses using enriched NK cells are needed to evaluate the increase in \textit{PRF1} expression in terms of transcript copies/cell, and its relationship to NK cell heterogeneity in response to acute stress\textsuperscript{37–39}.

We found support for methylation as a mechanism for regulation of \textit{PRF1} expression, as there was a significant negative correlation between the average methylation of all 7 CpG sites in the \textit{PRF1} promoter and its expression. We observed this relationship at all TSST time points, when methylation levels were scored on a continuous scale or categorized into low or high status. Based on this, it is possible that the lack of a significant difference in average methylation of all 7 CpG sites between CFS and NF might contribute to the lack of difference in \textit{PRF1} expression between CFS and NF in this study. In general, our results suggest methylation could be an important epigenetic determinant of inter-individual differences in \textit{PRF1} expression and thus the average methylation of all 7 CpG sites may account for the reported discrepancies (increase, decrease or no change) in \textit{PRF1} expression among different CFS studies\textsuperscript{7–9}. These results further imply the importance of measuring both \textit{PRF1} methylation and expression in understanding mechanisms of \textit{PRF1} expression in immune surveillance. Although there was no difference in methylation levels between CFS and NF, these groups showed some differences in the relationship between methylation and expression, immediately following TSST (1:50 pm) and at the last time point following TSST (3:05 pm). It appears that at 1:50 pm following TSST, a subset of CFS subjects with high baseline methylation (10:00 am methylation) in the MSR contributed to the blunted response in the \textit{PRF1} expression in comparison to NF subjects (compare Figs. 2B and 5B). At the last time point following TSST (3:05 pm) when \textit{PRF1} expression significantly increased in CFS (both in whole blood and PBMCs), its negative relationship with methylation at its closest time (3:05 pm) became non-significant. A mechanistic explanation appears complex for these disease-specific differences observed in this study. We focused on the relationship between \textit{PRF1} promoter methylation and mRNA, but many other mechanisms may be involved to regulate the expression of \textit{PRF1}, including a GRE located between –720 and –691 (Fig. 1) and the IL-2 responsive regulation of upstream enhancers in the \textit{PRF1} promoter\textsuperscript{40,41}.

Figure 5. Differential expression of \textit{PRF1} based on low versus high methylation status of its promoter in the MSR. Subjects were grouped into low or high methylation status based on the median split (65%) of the average 10:00 am methylation of 7 CpG sites in the MSR in all subjects. (A) \textit{PRF1} expression in whole blood (average ± SEM) in subjects grouped into low (dotted line) versus high (solid line) methylation at all TSST time-points. Gray vertical bar designates time of speech and math challenge in TSST. Subjects with low methylation had significantly higher expression than subjects with high methylation at all TSST time-points (\(P = 0.0002–0.05\)). (B) Histograms show the whole blood \textit{PRF1} expression immediately following TSST (1:50 pm) in CFS and NF subjects categorized into low and high methylation. Notes: \(P\)-values above gray brackets indicate significant differences in \textit{PRF1} expression between low versus high methylation subjects within CFS and NF. \(P\)-value above the black bracket indicates significant difference in \textit{PRF1} expression between CFS and NF subjects with high methylation.
leng paradigm. Study participants were screened for medication and no participant was on immunosuppressors. However, the impact of medications on HPA axis responsivity, if any, is unknown. Although statistically significant, the observed differences (2.6%–6.8%) in CpG site-specific methylation in the MSR between the pre- (10:00 am) and post (3:05 pm) TSST samples were small, possibly confounded due to the mixture of cell types present in PBMC. The methylation profile of the PRF1 MSR in this study (81 subjects) differed compared to an earlier study (5 subjects), possibly due to a substantial difference between these studies in the number of samples. One of the important questions, however, is whether the small percentage changes in methylation observed in this study are likely to be an important biological mechanism for regulating PRF1 expression. Some recent studies suggest that small changes in DNA methylation (2%–10%) can indeed translate to larger changes (1.5- to 32-fold) in gene expression. While these reported estimates of the impact of DNA methylation on expression vary considerably depending on the gene, tissue, environment and statistical analysis, our estimate of a 3% increase in PRF1 expression with every 1% decrease in methylation agrees with the estimate of impact of FXN methylation on its expression. These results, although limited to cross-sectional studies, support the view that subtle epigenetic changes can influence gene expression in response to environment.

Conclusion

In conclusion, we have documented an increase in PRF1 expression that parallels an increase in NK cells in response to acute psychosocial stress where patients with CFS had a blunted response compared to NF controls. Blunted expression by CFS may be related to high baseline PRF1 promoter methylation that was found to be an important epigenetic determinant of inter-individual differences in PRF1 expression. Further studies are needed to confirm these results and to evaluate explanations for the observed dynamics of PRF1 expression. It will also be interesting to investigate the signal transduction events resulting in peripheral influx of NK cells, as well as PRF1 expression and its functional role in the context of acute stress, and to identify molecular mechanisms that may be shared between stress and infection.

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Author Contributions

VRF, MSR and ERU conceived and designed the experiments. VRF and JM conducted the experiments. VRF and MSR analyzed the data. VRF wrote the first draft of the manuscript. VRF, MSR, TAW and ERU contributed to manuscript writing and revision. All authors reviewed and approved the final manuscript.

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