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Impact of acute psychosocial stress on peripheral blood gene expression pathways in healthy men☆

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ABSTRACT

We investigated peripheral blood mononuclear cell gene expression responses to acute psychosocial stress to identify molecular pathways relevant to the stress response. Blood samples were obtained from 10 healthy male subjects before, during and after (at 0, 30, and 60 min) a standardized psychosocial laboratory stressor. Ribonucleic acid (RNA) was extracted and gene expression measured by hybridization to a 20,000-gene microarray. Gene Set Expression Comparisons (GSEC) using defined pathways were used for the analysis. Forty-nine pathways were significantly changed from baseline to immediately after the stressor ($p < 0.05$), implicating cell cycle, cell signaling, adhesion and immune responses. The comparison between stress and recovery (measured 30 min later) identified 36 pathways, several involving stress-responsive signaling cascades and cellular defense mechanisms. These results have relevance for understanding molecular mechanisms of the physiological stress response, and might be used to further study adverse health outcomes of psychosocial stress.

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1. Introduction

The importance of psychosocial stress as a risk factor for a variety of negative health outcomes is widely recognized. Stress induces endocrine and immune system responses that help coping with the change in demand (McEwen, 1998) and stressors exert these influences by regulation of genes. Findings from previous gene expression studies in animal models show changes due to acute stress exposure cannot be readily translated to the human paradigm (Fujikawa et al., 2000; Liberzon et al., 1999). It has been suggested that peripheral blood mononuclear cell (PBMC) gene expression may provide an indicator of gene activation changes as a response to stress in humans (Segman et al., 2005), as lymphocytes have been shown to be perturbed following acute psychological stress (Aloe et al., 1994), probably mediated through endocrine and immune markers that are activated by the stressor. Only very few studies have investigated gene expression alterations due to stress in humans. Studies examining genome-wide transcriptional activity in PBMCs have been reported. One examined chronic isolation and showed it was associated with specific signaling pathways involved in immune regulation and biological stress responses (Cole et al., 2007). A more recent study associated chronic care-giving stress with alterations in glucocorticoid signal transduction as reflected by altered gene expression compared to a control group (Miller et al., 2008). Microarrays, which allow high-throughput profiling of transcriptional activity, are powerful tools that detect signatures of biological processes that underlie adaptive and maladaptive responses to a challenge of the system. Measurement of expression of multiple genes or even genome-wide transcriptional activity patterns is warranted. Previous studies have not attempted to study gene expression alterations using more than one single measurement time point. However, considering the complex chronology of biological processes that occur due to exposure to an acute stressor, repeated measurement of gene expression is crucial. In the current pilot study, we attempted to repeatedly measure gene expression patterns using a genome-wide approach allowing us to examine biological pathways changes that follow an acute psychosocial laboratory stressor in adult healthy men. As this is a "proof of concept" approach that will drive future research endeavors using a repeated measurement approach, no hypotheses about

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alterations in specific pathways were formulated. Therefore, the following analyses should be regarded as explorative in nature and will hopefully inform future decisions on formulating specific hypotheses. However, we expected to find changes in processes that are relevant for the biological response to stress. In particular, psychological stress is known to activate the hypothalamic–pituitary–adrenal (HPA) axis (Charmandari et al., 2005). The HPA axis plays a fundamental role in regulating other homeostatic systems, including the sympathetic nervous system and the immune system. Cortisol exerts inhibitory effects on the secretion of pro-inflammatory cytokines, including interleukin (IL)-6, and helps return these cytokines to baseline levels after stress. Increased sympathetic responses may mediate increased immune responses especially during stress. Thus, we expected changes in gene expression profiles that were closely related to changes in these biological stress systems.

2. Methods

2.1. Participants and conditions

This study was approved by the Institutional Review Boards of Emory University School of Medicine and the Centers for Disease Control and Prevention (CDC). All subjects were recruited via newspaper advertisement. Only male participants were included in the study. Exclusionary criteria were significant medical illness, past or current presence of psychotic symptoms or bipolar disorder, current presence of psychoactive substance abuse/dependency or eating disorders, as well as hormonal and psychotropic medication in the 4 weeks prior to study entry. Written informed consent was obtained prior to study entry and subjects were admitted as inpatients to the Emory University General Clinical Research Center. This study was conducted as part of a larger study.

2.2. Psychosocial stress procedure

A standardized and validated psychosocial stress test, the Trier Social Stress Test (TSST) (Kirschbaum et al., 1993), was performed between 15:00 h and 16:00 h. The test consists of a 10-min anticipation and preparation phase and a subsequent 10-min public speaking and mental arithmetic task in front of three individuals.

2.3. Assessments during the stress procedure

2.3.1. Psychometric measures

We measured positive and negative affect before and after TSST using the Positive and Negative Affect Scale (PANAS) (Crawford and Henry, 2004), which comprises 20 items describing emotions experienced at the present moment.

2.3.2. Biological measures

At 12:00 h, an intravenous (IV) catheter was inserted and subjects were not allowed to intake anything per oral except for water until 17:00 h. Blood was sampled in ethylenediaminetetraacetic acid (EDTA) tubes through the IV before (−15 and 0 min), during (15 min) and after the stress exposure (30, 45, 60, 75, and 90 min) for assessment of plasma adrenocorticotropin (ACTH), cortisol, norepinephrine/epinephrine, and interleukin (IL)-6 concentrations. ACTH and cortisol concentrations were measured using commercial radioimmunoassays (ACTH: Nichols Institute, San Juan Capistrano, CA; cortisol: DiaSorin Corporation, Stillwater, MN). Inter- and intra-assay coefficients of variation were less than 6% for ACTH and less than 4% for cortisol. Catecholamines were measured by reverse-phase, ion pair high performance liquid chromatography. For norepinephrine, mean intra-assay coefficient of variation was 7.1% (>800 pg/ml) and mean inter-assay coefficient of variation was 10.3% (300–550 pg/ml). For epinephrine, mean intra-assay coefficient of variation was 9.6% (>80 pg/ml) and mean inter-assay coefficient of variation was 16.3% (60–140 pg/ml). Plasma IL-6 concentrations were measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

2.3.3. Gene expression profiling

Whole blood was drawn (at 0, 30, and 60 min) into an 8 ml BD Vacutainer Cell Preparation Tube with sodium citrate (Becton Dickinson, NJ), and immediately processed according to manufacturer's instructions. Total ribonucleic acid (RNA) was isolated using TRIzol™ reagent (Invitrogen, CA) and RNA quality and quantity was assessed using the Agilent 2100 Bioanalyzer, all RNA used had RIN >8.5. Contaminating deoxyribonucleic acid (DNA) was removed by incubation with 1 U DNaseI (GeneHunter Corp., TN) at 37 °C for 15 min. RNA was labeled and arrays processed as previously described (Ojaniemi et al., 2003; Whistler et al., 2003). Hybridization was onto a 20K array (MWG Biotech, Germany, now Ocimum Biosolutions). A median background value was calculated around each of the features and subtracted from the mean feature signal to give the net signal for the respective gene. The data was quantile normalized and $\log_2$ transformed. Features showing minimal variation across the set of arrays were excluded from the analysis, those whose expression differed by at least 1.5-fold from the median in at least 50% of the arrays were retained, giving 7247 genes for further analysis.

2.4. Statistical analyses

2.4.1. Gene set expression comparison (GSEC) analysis

The GSEC tool (Xu et al., 2008) in BRB-ArrayTools (v3.7; http://linus.nci.nih.gov/BRB-ArrayTools.html) analyzes pre-defined gene sets for differential expression among pre-defined classes (Pavlidis et al., 2004). Gene sets for this analysis were derived from 593 BioCarta (http://www.biocarta.com), or 181 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (http://www.genome.jp/kegg/pathway.html) and 65 experimentally verified transcription factor targets. We identified pathways whose expression was differentially regulated among the classes. By analyzing gene sets, rather than individual genes, we were able to reduce the number of tests conducted, and allow findings among biologically related genes to reinforce each other. This analysis is different than annotating a gene list using gene ontology (GO) categories. For each gene set we computed the number of genes represented on the microarray in that group, and calculated the statistical significance for each gene by a univariate analysis using a paired *t*-test with a random variance model (Wright and Kirby, 2003). Then, for each gene set two *p*-value summary statistics (the Fisher (LS) and the Kolmogorov–Smirnov (KS) statistics (Simon and Lam, 2004) and the Efron–Tishirani's maxmean test statistic (Efron and Tishirani, 2007) were calculated. The significance level associated with the gene set is the proportion of the random samples giving as large a value of the summary statistic as in the actual genes of the specified gene set. We considered a gene set to be significantly differentially regulated if one of the tests was <0.05.

2.4.2. Transcription factor analysis

In an attempt to determine the over-representation of transcription factor binding sites (TFBS) within the set of co-expressed genes from the GSEC pathway analysis, as compared with a background set of genes, the Opossum system was used for enrichment analysis (Ho Sui et al., 2005; Huang et al., 2005). The analysis parameters looked at promoters 2000 nucleotides upstream of each gene's transcription start site, using a conservation level of top 10 percentile of non-coding conserved regions with an absolute minimum percent identity of 70%. Two measures of statistical over-representation were measured, a *Z*-score and a one-tailed Fisher exact probability.

2.4.3. Peripheral markers analysis

The Friedman test was used to compare observations repeated on the same subjects.

2.5. Microarray validation

2.5.1. Quantitative real-time polymerase chain reaction (qPCR)

Validation of the microarray gene expression findings was performed using qPCR on four genes that showed different expression patterns between the three time points for all donors. The candidate genes for validation are given in Table 1.

Endogenous control genes were selected on the basis of the microarray gene expression data from all time points. Ten genes were selected that showed low coefficients of variation, and genes with different functions were chosen to avoid genes belonging to the same biological pathways that might be co-regulated. GeNorm was used to select the most stable pair-wise combination of reference genes: phosphoglycerate kinase 1 (PGK1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). The slope of a five-step fivefold dilution standard curve (100 μg–10 pg) for each primer set was used to determine their PCR amplification efficiency ($10^{-1/\text{slope}}$). The template cDNA was pooled total RNA extracted from PBMC of several donors.

One microgram of DNAse digested RNA was reverse transcribed into cDNA using random hexamers and the Transcriptor Reverse Transcriptase kit (Roche Applied Sciences, Indianapolis, IN) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the LightCycler® 480 system and the Probes Master Mix kit (Roche) in 96 well plates according to the manufacturer's instructions. The primers and probes used are outlined in Table 1. All samples including the external standards and non-template control were run in duplicate. The cycling conditions consisted of one cycle at 95 °C for 5 min followed by 45 cycles of 95 °C × 15 s, 60 °C × 15 s and 72 °C × 45 s. The Lightcycler 480 software was used to extract crossing points. The data was normalized to GAPDH and PGK1 using the geometric means of the reference genes.

3. Results

Demographic characteristics of the participants are summarized in Table 2. Positive affect did not change before and after stress. However, negative affect increased significantly from pre- to post-stress.