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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.

Table 1
Hydrolysis primer and probe sets used for the validation of gene expression data. The probes were labeled with the HEX fluorophore 5′, and the non-fluorescent Black Hole Quencher 3′.

Gene symbol	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Probe sequence (5′–3′)	Amplicon length (bp)	PCR efficiency[a]
GAPDH	AACCTGCCAAATATGATGACATCA	GCCCAGGATGCCCTTGA	AGCAGGCGTCGGAGGGCCC	67	1.88
PGK1	CAAGAAGTATGCTGAGGCTGTCA	CAAATACCCCCACAGGACCAT	TCGGGCTAAGCAGATTGTGTGG	68	1.93
PKC	TGACAAACCCCCGTTCTTGA	GTTGACATATTCCATGACGAAGTACA	ACTCCTGCTTCCAGACAGTGGATCGG	82	1.80
CASP8	CCTGGGTGCGTCCACTTT	CAAGGTTCAAGTGACCAACTCAAG	TGGGCACGTGAGGTTGGGCC	78	1.96
ALDH3A2	GCAGCGATTTGACCACATTTTC	TAACATGGACTTTTCCCTCCCA	CGGTTGGCAAAATTGTCATGGAAGCT	120	1.88
IL6R	GTACCACTGCCCACATTCCT	CAGCTTCCACGTCTTCTTGA	CCTGGCCTTCGGAACGCTCCTC	71	1.99

[a] The maximum efficiency for a PCR reaction = 2.

Analysis of repeated measures of peripheral markers revealed significant differences between time points for ACTH ($\chi^2(7) = 24.8$; $p = 0.001$), cortisol ($\chi^2(7) = 34.5$; $p < 0.001$), norepinephrine ($\chi^2(7) = 47.7$; $p < 0.001$), and epinephrine ($\chi^2(7) = 41.0$; $p < 0.001$) (Fig. 1A–D). Plasma IL-6 ($\chi^2(7) = 38.2$; $p < 0.001$) and $\log_2$ normalized signal intensity for IL-6 extracted from gene expression data are presented in Fig. 2.

Comparison between baseline (0 min) and stress samples (30 min) revealed a total of 49 pathways that significantly changed ($p < 0.05$) (Table 3). When comparing stress (30 min) and recovery samples (60 min), 36 pathways were significantly changed (Table 4). The parametric p-values and the geometric means for all genes identified in the pathways of Tables 3 and 4 can be found in Supplementary file 1. Eight pathways were identified as common to each of these analyses (bold highlight, Tables 3 and 4) involving 210 unique genes. This represents 36 and 45% of the unique genes common to these eight pathways. We are able to dissect out the gene expression changes in each of the pathways (Supplementary file 2). The Janus kinase-signal (JAK/STAT) signaling pathway is central to the stress response and looking at the expression changes between baseline and stressor (0 and 30 min) we find 42/56 genes are showing ≥2-fold increase in expression. Then 31/42 of these genes are down-regulated (≥2-fold change) during the recovery (30–60 min). A further 11 genes in this pathway, unchanged from baseline to stress, are also down-regulated during the recovery. Similar analytical dissection of the gene regulation for the remaining seven common pathways is given in Supplementary file 2.

The transcription factors that regulate the genes in the pathways that were found to be differentially expressed were examined by over-representation analysis (Tables 5 and 6).

The limited amount of RNA on several subjects allowed for validation of four genes by qPCR (Fig. 3). Overall there was very good agreement of the qPCR data with the mRNA generated microarray data, in that the trends of differential expression were maintained in both.

Table 2
Demographic features and psychological measures during psychosocial stress test.

	$n = 10$
Mean age in years (range)	32.4 (20–54)
Mean BMI (range)	25.8 (21.3–33.4)
Race (%)	
Caucasian	5 (50)
African-American	5 (50)
PANAS-positive	
Pre-stress (95% CI)	18.8 (12.5–25.0)
Post-stress (95% CI)	16.8 (9.5–24.0)
PANAS-negative	
Pre-stress (95% CI)	3.2 (−0.8 to 7.2)
Post-stress (95% CI)	10.8* (5.9–15.6)

BMI = body mass index; PANAS = Positive Affect Negative Affect Scale.
* $p < 0.01$.

4. Discussion

Acute stressors trigger adaptive up-regulation of natural immunity, redistribution of cells (Goebel and Mills, 2000), and preparation of the natural immune system (Miller et al., 2005). In our analysis these effects from psychosocial stress are reflected in changes in acute inflammatory response pathways (see Table 3, 33–36), and pathways regulating adhesion molecules (Table 3, 1–5). The effects of the leukocytosis are more apparent in the baseline-stress analysis than the stress–recovery time period, as a greater number of pathways are associated with cell migration and adhesion in the former compared to the latter. In addition, stress response pathways associated with nuclear factor kappa B (NF-κB) appear to be associated with the stress–recovery period (Table 4, 29–32). A variety of pathways were significantly changed between baseline and stress, involving basic cell regulatory and signaling processes, many involving cell growth, differentiation and proliferation (Table 3, 17–29). Also, it was noted that ras-related C3 botulinum toxin substrate 1 (RAC1) was central to the functioning of several pathways in the baseline–stress analysis (Table 3, 1, 2 and 16). This protein is a pleiotropic regulator of many cellular processes, including the cell cycle, cell–cell adhesion, and motility (through the actin network). Thus, all these pathways are implicated in the adhesion and diapedesis of immune cells from the lymphatic system to the blood stream. A shortfall in this pilot study was that we did not monitor the cell subsets during the course of the time series, which is essential for larger scale studies, so we can account for the differences in the gene expression profiles being driven by the changes in T cell, B cell and natural killer cell populations.

Acute stressors trigger suppression of specific immunity (Segerstrom and Miller, 2004). The role of psychological stress in the regulation of lymphocyte adhesion molecules has been well examined (Mills et al., 1998; Redwine et al., 2003). These mechanisms are also reflected by changes in the pathway implicated in adhesion (Table 3, 1–5). More specifically, stress induces changes in effector T cell distribution, as can be seen by changes in the pathways involved in T helper cell mobilization (Table 3, 28 and 34–35).

Apoptosis plays an important role in the organism's response to acute stress. While lymphocytes expand and proliferate in response to acute stress, apoptosis has to be inhibited in the early phase of the stress response. In the later stages, though, apoptosis must occur to reduce the number of accumulated cells and to restore homeostasis. Our findings show that during stress gene products involved in apoptotic processes are altered upon exposition of the organism to acute stress (Table 3, 6–7, 15 and 23).

Several pathways involved in the immediate stress response were common across stress and recovery, indicating common mechanisms underlying the processes that allow the body to mount an adequate response and recover from a stressor to regain homeostasis. Two of the pathways, involved in metabolic processes (Oxidative Phosphorylation and Ascorbate metabolism, a total of 52 genes) all showed up-regulation of gene expression

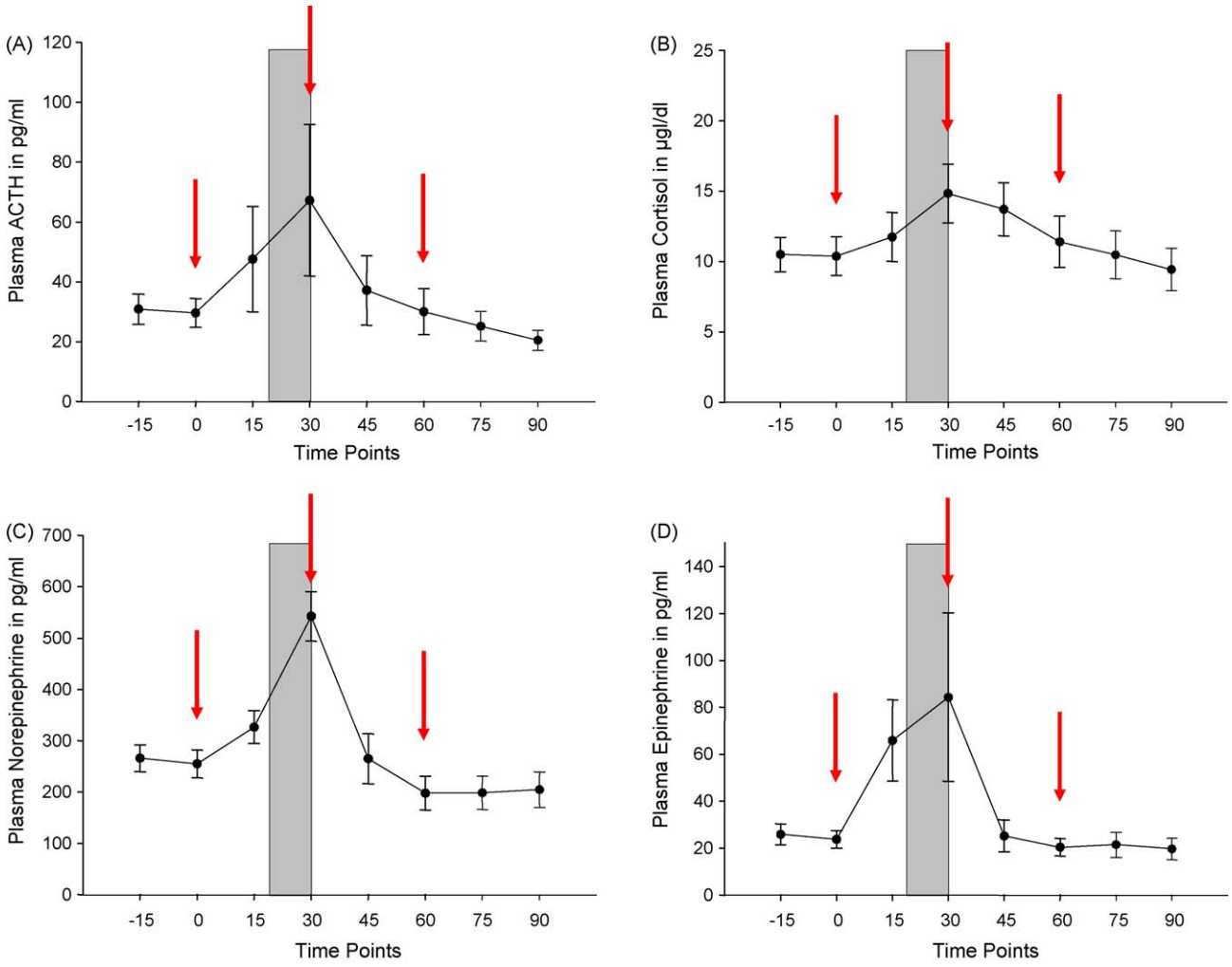

Fig. 1. Biological measures during psychosocial stress test. Plasma ACTH (A), plasma cortisol (B), plasma norepinephrine (C), and plasma epinephrine (D) changed significantly over time. Red arrows indicate when blood sample was analyzed for PBMC gene expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

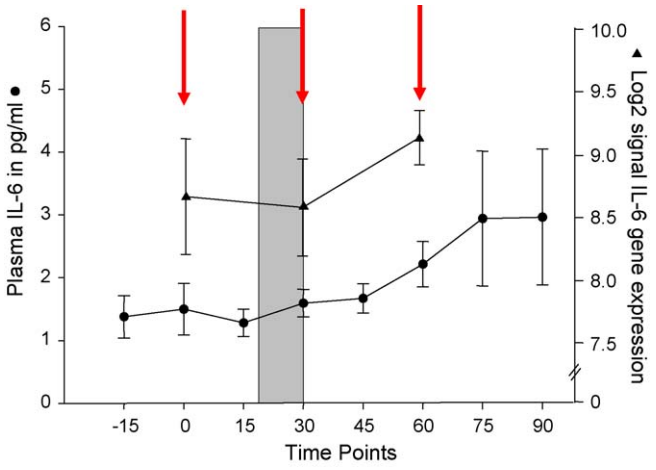

Fig. 2. Plasma IL-6 and log2 normalized signal intensity for IL-6 extracted from gene expression data. Similar profiles for the two measures add validity to the gene expression data. Red arrows indicate when blood sample was analyzed for PBMC gene expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

between baseline-stress, followed by down-regulation during recovery, as did the vast majority of genes in the JAK/STAT signaling pathway. The immediate physiological consequences of the stress response are evident in the analyses seen here, i.e. immune activation and energy mobilization. However, genes in the three pathways associated with the cell cycle, cell differentiation, etc., showed mixed regulation of the genes, and genes that were up- (or down-) regulated in the baseline-stress analysis were still up-regulated in the stress–recovery analysis (Supplementary file 2). Some of this reflects the biological changes occurring in the lymphocyte populations with respect to cell differentiation, activation and survival, as the proportion and numbers of circulating cells changes, as is well documented during stressors (Segerstrom and Miller, 2004; Zorrilla et al., 1995).

There are five transcription factors that are common to the genes in both GSEC pathway analyses (Tables 5 and 6). These are NF-κB, V-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian) (RELA), nuclear receptor subfamily 3, group C, member 1 (NR3C1 or glucocorticoid receptor), spermatogenic leucine zipper 1 (SPZ1), and E74-like factor 5 (ELF5). The first two interact with each other to form the NF-κB transcription complex. This complex is involved in cellular responses to stimuli such as

Table 3
Results of the paired class comparison analysis between baseline and stress time points using BioCarta and KEGG pathways as defined gene sets. Pathways containing more than five genes present on the array were included in the analysis. This meant that 176 BioCarta and 146 KEGG pathways were considered. A univariate analysis using paired *t*-tests with a random variance model was run to determine the differentially expressed genes in each gene set. Significant gene sets were then identified with summary statistics (the LS/KS permutation test and Efron–Tishirani's maxmean test (Efron and Tishirani, 2007) using a threshold of 0.05 for at least one test. In the combined BioCarta/KEGG analysis 49 pathways passed this threshold. Pathways in highlighted in bold are common to both the baseline-stress and stress–recovery analyses. *p*-Values and Log ratios are presented in Appendix BSupplementary file 1.

	Pathway ID	Pathway description	No. of genes	Biological function
1	h_ucalpain	uCalpain and friends in Cell spread	11	Adhesion
2	h_agr	Agrin in postsynaptic differentiation	19	Adhesion, cell migration
3	h_ecm	Erk and PI-3 kinase are necessary for collagen binding in corneal epithelia	10	Adhesion, cell migration
4	h_rho	Rho cell motility signaling pathway	13	Adhesion, cell morphology
5	h_rac1	Rac 1 cell motility signaling pathway	10	Adhesion, cell signaling
6	**hsa05040**	**Huntington's disease**	**13**	**Apoptosis**
7	h_ras	Ras signaling pathway	8	Apoptosis
8	h_intrinsic	Intrinsic prothrombin activation pathway	6	Blood collection
9	hsa04110	Cell cycle	43	Cell cycle
10	h_g1	Cell cycle: G1/S check point	9	Cell cycle
11	**h_skp2e2f**	**E2F1 destruction pathway**	**5**	**Cell cycle**
12	h_RacCycD	Influence of Ras and Rho proteins on G1 to S Transition	12	Cell cycle
13	**h_p27**	**Regulation of p27 phosphorylation during cell cycle progression**	**7**	**Cell cycle progression**
14	hsa04120	Ubiquitin mediated proteolysis	15	Cell cycle, immune response
15	h_pml	Regulation of transcriptional activity by PML	15	Cell growth, apoptosis
16	h_At1r	Angiotensin II mediated activation of JNK Pathway via Pyk2	16	Cell migration
17	h_mrp	Multi-drug resistance factors	6	Cell regulation
18	h_cardiacegf	Role of EGF receptor transactivation by GPCRs in cardiac hypertrophy	6	Cell regulation
19	hsa04540	Gap junction	35	Cell signaling
20	h_gpcr	Signaling pathway from G-protein families	10	Cell signaling
21	h_erk	Erk1/Erk2 MAPK signaling pathway	13	Cell signaling, cell growth and differentiation
22	h_calcineurin	Effects of calcineurin in keratinocyte differentiation	5	Cell signaling: Ca++
23	**hsa04630**	**JAK/STAT signaling pathway**	**56**	**Cell signaling: cytokines, growth factors, stress**
24	hsa04310	Wnt signaling pathway	48	Cell signaling: development, proliferation, mobility
25	h_pelp1	Pelp1 modulation of estrogen receptor activity	5	Cell signaling: growth factor signaling
26	**hsa04010**	**MAPK signaling pathway**	**114**	**Cell signaling: growth, inflammation, apoptosis**
27	h_fcer1	Fc epsilon receptor I signaling in mast cells	12	Cell signaling: immunity, arachadonic acid metabolism
28	hsa04330	Notch signaling pathway	17	Cell signaling: T-cell development
29	h_hes	Segmentation clock	8	Cell signaling: WNT and Notch
30	hsa00790	Folate biosynthesis	14	Cofactor biosynthesis
31	h_ppara	Mechanism of gene regulation by peroxisome proliferators via PPARa	20	Gene regulation
32	h_sppa	Aspirin blocks signaling pathway involved in platelet activation	6	Hemostasis
33	hsa04650	Natural killer cell mediated cytotoxicity	64	Immune response: NK cell
34	**h_dc**	**Dendritic cells in regulating TH1 and TH2 development**	**5**	**Immune response: T-cell**
35	h_tcr	T cell receptor signaling pathway	14	Immune response: T-cell
36	h_mef2d	Role of MEF2D in T-cell apoptosis	5	Immune response: T-cell; apoptosis
37	hsa04730	Long-term depression	32	Learning and memory
38	hsa04720	Long-term potentiation	28	Learning and memory
39	hsa00380	Tryptophan metabolism	41	Metabolism: Amino acid
40	hsa00530	Aminosugars metabolism	6	Metabolism: carbohydrate
41	**hsa00053**	**Ascorbate and aldarate metabolism**	**9**	**Metabolism: carbohydrate**
42	**hsa00190**	**Oxidative phosphorylation**	**43**	**Metabolism: energy production**
43	hsa00770	Pantothenate and CoA biosynthesis	8	Metabolism: fatty acid
44	hsa00531	Glycosaminoglycan degradation	10	Metabolism: glucosamine
45	hsa00534	Heparan sulfate biosynthesis	7	Metabolism: glucosamine
46	hsa00604	Glycosphingolipid biosynthesis - ganglioseries	9	Metabolism: glycolipid
47	hsa00960	Alkaloid biosynthesis II	16	Metabolism: secondary metabolites
48	h_prc2	PRC2 complex long-term gene silencing by modification of histone tails	7	Regulation gene expression
49	h_arap	ADP-ribosylation factor	10	Vesicular trafficking

stress and plays a key role in regulating the immune response (Avitsur et al., 2003; Bhatara et al., 1998). In fact, it has recently been identified as a potential critical bridge between stress and cellular activation (Bierhaus et al., 2003; Rosecrans and Karin, 1998). Another common transcription factor regulating the differentially expressed genes in the common pathways was NR3C1, a transcription factor that binds to glucocorticoid response elements (GRE) and is a modulator of other transcription factors. It affects inflammatory responses, cellular proliferation and differentiation. SPZ1 is involved in regulating genes involved in cell growth and differentiation, while ELF5 is an ETS-domain transcription factor, which regulate a diverse range of biological functions including mammalian hematopoiesis (Sharrocks et al., 1997).

Cortisol activation of the glucocorticoid receptor (GR) exerts broad anti-inflammatory effects by inhibiting NF-κB/Rel transcription factors and other pro-inflammatory signaling pathways such as the JAK/STAT signaling pathway (Collado-Hidalgo et al., 2006; Rhen and Cidlowski, 2005). Exploring the JAK/STAT signaling pathway in the baseline-stress GSEC analysis (Supplementary file

Table 4
Results of the paired class comparison analysis between stress and recovery time points using BioCarta and KEGG pathways as defined gene sets. Pathways containing more than five genes present on the array were included in the analysis. This meant that 176 BioCarta and 146 KEGG pathways were considered. A univariate analysis using paired *t*-tests with a random variance model was run to determine the differentially expressed genes in each gene set. Significant gene sets were then identified with summary statistics (the LS/KS permutation test and Efron–Tishirani's maxmean test (Efron and Tishirani, 2007) using a threshold of 0.05 for at least one test. In the combined BioCarta/KEGG analysis 49 pathways passed this threshold. Pathways in highlighted in bold are common to both the baseline-stress and stress–recovery analyses. *p*-Values and Log ratios are presented in Supplementary file 1.

	Pathway ID	Pathway description	No. of genes	Biological function
1	hsa04810	Regulation of actin cytoskeleton	89	Adhesion
2	**hsa05040**	**Huntington's disease**	**13**	**Apoptosis**
3	**h_p27**	**Regulation of p27 phosphorylation during cell cycle progression**	**7**	**Cell cycle progression**
4	**h_skp2e2f**	**E2F1 destruction pathway**	**5**	**Cell cycle progression, TF**
5	h_gsk3	Inactivation of Gsk3 by AKT: accumulation of b-catenin in macrophages	8	Cell proliferation/differentiation
6	h_CCR3	CCR3 signaling in eosinophils	14	Cell signaling
7	h_akt	AKT signaling pathway	5	Cell signaling: cell survival
8	**hsa04630**	**JAK/STAT signaling pathway**	**56**	**Cell signaling: cytokines, growth factors**
9	**hsa04010**	**MAPK signaling pathway**	**114**	**Cell signaling: growth, inflammation, apoptosis**
10	hsa04912	GnRH signaling pathway	38	Cell signaling: HPG axis
11	hsa04620	Toll-like receptor signaling pathway	45	Cell signaling: immunity
12	hsa04664	Fc epsilon RI signaling pathway	36	Cell signaling: immunity, arachadonic acid metabolism
13	h_il1r	Signal transduction through IL1R	15	Immune response, cell signaling
14	h_tall1	TACI and BCMA stimulation of B cell immune responses.	7	Immune response: B-cell
15	**h_dc**	**Dendritic cells in regulating TH1 and TH2 development**	**5**	**Immune response: T-cell**
16	hsa00460	Cyanoamino acid metabolism	5	Metabolism: amino acid
17	hsa00340	Histidine metabolism	27	Metabolism: amino acid
18	hsa00450	Selenoamino acid metabolism	12	Metabolism: amino acid
19	hsa00220	Urea cycle and metabolism of amino groups	8	Metabolism: amino acid
20	**hsa00053**	**Ascorbate and aldarate metabolism**	**9**	**Metabolism: carbohydrate**
21	hsa00051	Fructose and mannose metabolism	17	Metabolism: carbohydrate
22	hsa00010	Glycolysis/gluconeogenesis	18	Metabolism: carbohydrate
23	hsa00030	Pentose phosphate pathway	6	Metabolism: carbohydrate
24	**hsa00190**	**Oxidative phosphorylation**	**43**	**Metabolism: energy**
25	hsa00591	Linoleic acid metabolism	17	Metabolism: fatty acid
26	hsa00600	Sphingolipid metabolism	14	Metabolism: fatty acid
27	h_eicosanoid	Eicosanoid metabolism	5	Metabolism: fatty acid. Stress response
28	hsa00940	Stilbene, coumarine and lignin biosynthesis	7	Metabolism: xenobiotic
29	h_eponfkb	Erythropoietin mediated neuroprotection through NF-κB	7	NF-κB
30	h_hcmv	Human cytomegalovirus and Map kinase pathways	8	NF-κB
31	h_nthi	NF-κB activation by non-typeable hemophilus influenzae	18	NF-κB
32	h_arenrf2	Oxidative stress induced gene expression via Nrf2	9	Stress response
33	hsa03020	RNA polymerase	10	Transcription
34	h_eif4	Regulation of eIF4e and p70 S6 kinase	14	Translation regulation
35	hsa04130	SNARE interactions in vesicular transport	13	Vesicular trafficking
36	h_SARS	SARS coronavirus protease	8	Glycolysis

Table 5
Statistically over-represented TFBS in genes associated with the GSEC baseline and stress analysis as determined by Opossum. Results displayed where *Z*-score ≥5 and Fisher ≤0.01.

Transcription factor	TF class	Information content[a]	Target gene hits	Background TFBS rate[b]	Target TFBS rate[b]	*Z*-score[c]	Fisher score[d]
MZF1_5-13	Zn-Finger, C2H2	9.4	301	0.0222	0.0245	10.6	2.71E−07
MZF1_1-4	Zn-Finger, C2H2	8.6	377	0.0464	0.0494	10.3	2.20E−04
SP1	Zn-Finger, C2H2	9.7	290	0.02	0.022	9.9	6.78E−08
TEAD1	TEA	15.7	100	0.0029	0.0037	9.9	2.07E−04
RELA	REL	14.8	141	0.0039	0.0048	9.2	2.39E−05
GABPA	ETS	13.9	157	0.0049	0.0057	8.3	8.50E−05
REL	REL	10.5	227	0.0089	0.01	8.1	4.86E−07
CEBPA	bZIP	9.2	210	0.0118	0.013	7.8	5.60E−04
ELF5	ETS	8.7	353	0.0291	0.0307	6.6	4.05E−07
Spz1	bHLH-ZIP	11.9	166	0.0049	0.0056	6.6	1.13E−08
NR3C1	Nuclear Receptor	14.7	60	0.0023	0.0027	6.4	1.48E−03
ELK1	ETS	8.8	315	0.0201	0.0213	6.1	2.56E−07
HLF	bZIP	11.1	124	0.0048	0.0054	5.9	6.55E−03
Arnt-Ahr	bHLH	9.5	319	0.0179	0.0189	5.7	6.97E−05
GFI	Zn-Finger, C2H2	9.5	270	0.0169	0.0179	5.4	4.65E−05
RORA_1	Nuclear Receptor	13.2	197	0.006	0.0065	5.0	1.61E−09
NF-κB	REL	13.3	180	0.0057	0.0062	5.0	5.24E−07

[a] This is the specificity of the TFBS profile's position weight matrix.
[b] The number of times this TFBS was detected within the conserved non-coding regions of the background or target set of genes.
[c] The likelihood that the number of TFBS nucleotides detected for the included target genes is significant as compared with the number of TFBS nucleotides detected for the background set. *Z*-score is expressed in units of magnitude of the standard deviation.
[d] The probability that the number of hits vs. non-hits for the included target genes could have occurred by random chance based on the hits vs. non-hits for the background set.

Table 6
Statistically over-represented TFBS in genes associated with the GSEC stress and recovery analysis as determined by Opossum. Results displayed where *Z*-score ≥5 and Fisher ≤0.01.

Transcription factor	TF class	IC[a]	Target gene hits	Background TFBS rate[b]	Target TFBS rate[b]	*Z*-score[c]	Fisher score[d]
REST	Zn-Finger, C2H2	22.958	10	0.0002	0.0006	17.8	1.72E−03
NR3C1	Nuclear Receptor	14.749	49	0.0023	0.0031	9.9	2.77E−03
RELA	REL	14.757	105	0.0039	0.0047	7.1	3.93E−03
NF-κB	REL	13.345	142	0.0057	0.0065	6.2	2.39E−05
Spz1	bHLH-ZIP	11.907	127	0.0049	0.0056	5.8	9.30E−06
SRF	MADS	17.965	17	0.0005	0.0007	5.6	4.41E−02
ELF5	ETS	8.693	280	0.0291	0.0305	5.2	4.47E−05

[a] This is the specificity of the TFBS profile's position weight matrix.
[b] The number of times this TFBS was detected within the conserved non-coding regions of the background or target set of genes.
[c] The likelihood that the number of TFBS nucleotides detected for the included target genes is significant as compared with the number of TFBS nucleotides detected for the background set. *Z*-score is expressed in units of magnitude of the standard deviation.
[d] The probability that the number of hits vs. non-hits for the included target genes could have occurred by random chance based on the hits vs. non-hits for the background set.

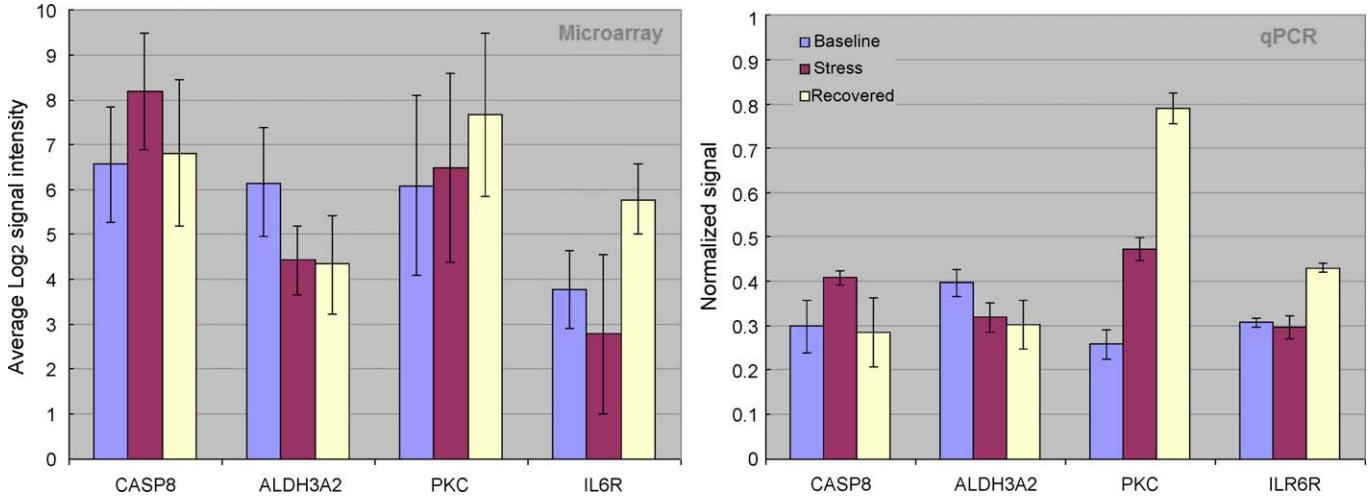

Fig. 3. Comparison of microarray gene expression (left) and qPCR data (right) for selected genes. Bar chart represents averaged signal ± SD.

3) we see 19 genes are identified with the NF-κB/RELA TFBS combination and eight genes are up-regulated ≥2-fold between baseline and stress, while only three of these genes are down-regulated. Three of five genes with NR3C1 (GR) promoter sequences in the JAK/STAT pathway are down-regulated (Supplementary file 3). We find that the cortisol effects are evident in the gene expression patterns during the time series analysis. This indicates by using state-of-the-art methodologies we can explore the biological changes of psychological stressors.

When blood is collected, the intrinsic pathway is activated by contact with the collection devices causing damage to the platelets and activation of factor XII (Schaffer et al., 2008). This pathway was identified as having differentially expressed genes in the baseline-stress analysis (Table 3).

Several limitations of the current study need to be addressed. The small sample size limits generalization to a broader population. Also, not all genes measured in the microarrays were annotated and genes that might be critical for the stress response might thus have been omitted from our analysis. Related to this issue, only a fraction of the annotated genes are summarized in a pathway. To some extent, we were able to address some of these limitations by analyzing gene sets, rather than individual genes, thus we were able to reduce the number of tests conducted, and allow findings among biologically related genes to reinforce each other. This functional class scoring is a more powerful method of identifying differentially expressed gene classes than the commonly used over-representation analysis, or annotation of gene lists based on individually analyzed genes (Simon and Lam, 2004). It indicates which gene sets contain more differentially expressed genes than would be expected by chance. Further, we were able to measure gene expression patterns at three time points (immediately before and after, as well as 30 min after an acute stressor) in our current study. It would have certainly been of great interest to have further measures beyond that time frame. Longer lasting changes in the endocrine, immune, and sympathetic nervous system could not be captured in our study. Clearly, future studies need to incorporate more measurement time points in order to be able to capture the complex biological processes that occur following an acute stressor. Also, increasing both sample size and sampling time points will allow us to more closely describe cellular events in a chronological order as they occur before, during, and after a stressor. Finally, we chose to use a within-subject measurement approach. This allowed us to examine changes of biological pathways after exposure to an acute stressor. However, further studies that will expand our current efforts will benefit from including a control condition in which no stress intervention occurred. This will allow discerning baseline and stress-related processes.

In conclusion, we were able to show even in a small sample of healthy volunteers that effects of acute psychological stress are reflected in genome-wide transcriptional activity changes in peripheral blood. Specific pathways may be changed in specific disorders in response to psychosocial stress. Our pilot study needs to be replicated and future studies will expand our current findings by including more subjects. The stress-related changes that we were able to describe are most likely adaptive responses to a

moderate stressor. It might be of great interest to use this approach in clinical samples. Identification of gene expression patterns relevant for psychiatric and somatic disorders that are related to stress has the potential to identify novel targets for the prevention and treatment of adverse outcomes of stress.

Conflicts of interest

Drs. Nater, Whistler, Vernon, and Ms. Mletzko and Mr Lonergan reported no biomedical financial interests or potential conflicts of interest. Dr. Heim reports having received funding from NIMH, CDC, NARSAD, ADAA, Eli Lilly, and Novartis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biopsycho.2009.06.009.

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