NON-TARGETED DATA ANALYSIS AS A MEANS OF IDENTIFYING COMMON TEMPORAL PATTERNS IN PLASMA METABOLITE LEVELS AND LEUKOCYTE TRANSCRIPTIONAL RESPONSE TO ENDOTOXEMIA

By

KIRSTEN ELIZABETH SLEIGHT

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ABSTRACT OF THE THESIS

Non-targeted Data Analysis as a Means of Identifying Common Temporal Patterns in Plasma Metabolite Levels and Leukocyte Transcriptional Response to Endotoxemia

by KIRSTEN ELIZABETH SLEIGHT

Thesis Director:

Ioannis P. Androulakis

An experimental procedure which has provided insight into organ, tissue, and cell response to systemic inflammation is the administration of lipopolysaccharide (LPS) bacterial endotoxin to healthy human volunteers. At low doses, LPS briefly mimics the effects of acute injury or systemic inflammation in altering physiologic and metabolic processes. In the case of endotoxemia, an understanding of its effects on the plasma metabolite concentrations is crucial, as metabolite levels affect the regulation of anti-inflammatory defenses by impacting important processes in immune cells. This study attempts to perform a complete metabolomic analysis of the changes in plasma metabolite composition after exposure to LPS. In healthy volunteers, LPS administration was observed to cause significant alterations in lipid and protein metabolism homeostasis within the first 6 hr following LPS. The use of untargeted bioinformatics-based exploration of the data allowed for the identification of the dominant patterns of response. Plasma lipid levels steadily increased during the 6 hr following the administration of a low dose of LPS, before reversing direction during the recovery phase. In contrast,

plasma levels of amino acids and amino acid derivatives decreased in the first 6 hr after LPS, before also reversing direction during the recovery phase.

Severe traumas are associated with hypercortisolemia due to both disruption of cortisol secretion rhythm and increase in its total concentration. Understanding the effects of altered cortisol levels and rhythms on immune function is of great clinical interest, to prevent conditions such as sepsis from complicating the recovery. By looking at transcriptional profiling of whole blood leukocytes in vivo, this study assessed their response to coupled rhythm and dose manipulation of cortisol levels preceding an immune challenge caused by the administration of LPS. The identification and clustering of probesets differentially expressed over time was used to correctly identify both the saline control group and the cortisol group without any prior knowledge of group assignments, and then to separate out three types of response to LPS exposure – cortisol suppressed, cortisol enhanced, and LPS dominated. The overall effect of the cortisol infusion seems to be an increase in the preparedness of immune cells to respond to potential threats, creating a preparatory effect to enhance the effectiveness of the immune response. These changes included an increase in the expression of genes coding for several cytokine and pattern recognition receptors, signal transduction elements, and receptor regulatory elements. Along with this, there was a decrease in the expression of genes that code for elements involved in protein translation, and for mitochondrial proteins. The increase in receptor and signal transduction protein expression indicated that the higher cortisol concentration served to prime the immune response, sensitizing cells to recognize and respond to potential infection or danger signals.

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INTRODUCTION

One of the experimental procedures which has provided insight into organ, tissue, and cell response to systemic inflammation is the administration of lipopolysaccharide (LPS) bacterial endotoxin to healthy human volunteers. At low doses, LPS briefly mimics the effects of acute injury or systemic inflammation in altering physiologic and metabolic processes (Lowry 2004) (Calvano and Coyle 2012). This model allows for the analysis of infectious stress response at various physiological levels, and has been used to develop and assess clinical therapies for the prevention or attenuation of systemic inflammatory response syndrome (SIRS) (Calvano and Coyle 2012).

A properly functional immune system is tied closely to the body's biological rhythms fluctuating in harmony. After trauma, infection, or other acute stress, the resulting inflammatory response disrupts these natural biological rhythms, including glucocorticoid (GC) secretion rhythms. These rhythms are an essential part of achieving homeostasis (de Kloet and Sarabdjitsingh 2008). An important GC involved in these rhythms is cortisol, which has been shown to be essential for the maintenance of homeostasis through mathematical modeling of the relevant regulatory mechanisms (Scheff, Calvano, et al. 2012). Previous studies have also established the role of GCs in the immune response (Barber, et al. 1993) (Yeager, Pioli and Guyre 2011), but these studies have focused on single immunological parameters.

The use of global transcriptomic studies allows for an increased understanding of the complex regulatory mechanisms which govern the inflammatory response (Calvano, et al. 2005) (Nguyen, Foteinou, et al. 2011), which is foundational to the development of semi-mechanistic models of inflammation (P. Foteinou, et al. 2009) (P. Foteinou, et al. 2011)

(Scheff, Mavroudis, et al. 2011). Along with these transcriptomic studies, metabolic alterations in response to endotoxemia can also provide important information for understanding systemic inflammation. In the case of endotoxemia, an understanding of its effects on the plasma metabolite concentrations is crucial, as metabolite levels affect the regulation of anti-inflammatory defenses by impacting important processes in immune cells (Pearce and Pearce 2013).

TEMPORAL METABOLIC PROFILING OF PLASMA DURING ENDOTOXEMIA IN HUMANS

One aspect of the response to endotoxemia involves metabolic alterations. The inflammatory response directs substrates towards splanchnic organs, and away from the periphery, while also introducing a catabolic state in order to meet increased demands for substrates and energy (Fong, et al. 1990) (Khovidhunkit, et al. 2004). This results in an alteration in plasma metabolite levels. While previous studies have examined individual changes in major metabolites caused by human endotoxemia (Fong, et al. 1990), an untargeted, bioinformatics-based exploration of the effects on plasma metabolite levels due to human endotoxemia is lacking.

An examination of the metabolic response as a whole is important when looking at systemic inflammation, as a change in the metabolic composition of a tissue is the result of regulation at several levels of cellular processes, including transcription, translation, and signal transduction. The various concentration of metabolites found in a sample at a particular time act as a sort of metabolic fingerprint that gives insight into the dominant regulatory mechanisms and the state of the body at that given time (Nicholson and Lindon 2008). Taking this unique information and combining it with a bioinformatics approach allows for a comprehensive picture of interactions within the system over time (Nicholson 2006) (Holmes, Wilson and Nicholson 2008). Because the production of metabolites is regulated and determined by complex processes beginning with transcription and moving on through translation and signal transduction, a better understanding of the metabolic response could offer insight into many clinical conditions. In the case of endotoxemia, an understanding of its effects on the plasma metabolite

concentrations is crucial, as metabolite levels affect the regulation of anti-inflammatory defenses by impacting important processes in immune cells (Pearce and Pearce 2013). This study attempts to perform a complete metabolomic analysis of the changes in plasma metabolite composition after exposure to LPS. Plasma samples were collected from healthy volunteers who had either received LPS or a placebo, and these samples were examined using untargeted biochemical profiling, to obtain temporal profiles for 366 metabolites at 5 time points in the 24 hr following treatment. The temporal profile data was then filtered to find significant changes in plasma metabolite levels. Further analysis pointed to lipid and protein metabolism as playing prominent roles in regulation of the body's response to systemic inflammation, as well as highlighting their opposing dynamics in this regulation.

METHODS

Human plasma samples

For this proof-of-principle study, archived flash frozen blood plasma samples from 19 healthy subjects were used. Of the subjects, 15 had been given a bolus dose of National Institutes of Health (NIH) Clinical Center Reference Endotoxin, as previously described (Alvarez, et al. 2007) (Jan, Coyle and Oikawa, et al. 2009) (Jan, Coyle and Macor, et al. 2010). For the control group, 4 subjects were given saline. Blood samples for both groups were taken at t = 1, 2, 6, and 24 hr, and stored at -80°C prior to analysis.

Biochemical profiling of plasma samples

Metabolomic analysis was carried out by Metabolon (Durham, NC, USA) according to previously published methods (Evans, et al. 2009). Samples were prepared by using methods to achieve maximum recovery of small molecules, and the resulting extracts

were subjected to liquid or gas chromatography, before being analyzed using mass spectroscopy. The combination of the chromatography and mass spectroscopy data was used to identify the compounds through comparison to library entries of purified standards.

Data analysis

Once the metabolites had been identified, the dataset containing 366 metabolites with complete temporal profiles was analyzed. First, imputed and log transformed datasets were used to determine which metabolites exhibited differential temporal profiles between the LPS treated subjects and the control subjects. This was done using software designed for the extraction and analysis of differential gene expression (EDGE) (Leek, et al. 2006), with the significance threshold set at $p < 0.05$ and $q < 0.1$. Once the metabolites showing differential time profiles between LPS and control groups had been identified, they were processed using principal component analysis, and the averaged first principal components (PC1) were plotted against time for each treatment group. The significance of PC1 variance over time was determined for each treatment group using one way ANOVA, and Wilcoxon rank sum test with 1% significance level was used to compare PC1 values at each time point. Following this, the datasets which contained differential metabolites were concatenated into a single matrix, and consensus clustering ($p = 0.05$) was used to identify groups of metabolites with coherent temporal profiles (Nguyen, Nowakowski and Androulakis 2009). The biological significance of these profiles was determined based on the identities of the individual metabolites, and on metabolic pathways obtained from publicly available Kyoto Encyclopedia of Genes and Genomes

(KEGG) (Lissauer, et al. 2009), Human Metabolome Database (Wishart, et al. 2013), and Ingenuity Knowledge Base (Calvano, et al. 2005).

RESULTS

The aim of this study was to identify the major coherent patterns that emerge in the human plasma metabolome during the first 24 hr following systemic exposure to LPS. The study design (Figure 1) included two groups of subjects, with members of one group receiving a bolus dose of LPS at t = 0, and members of the other group receiving a saline injection at the same time point. Blood samples were collected at the 1, 2, 6, and 24 hr time points, and non-targeted biochemical profiling was used to determine metabolite response.

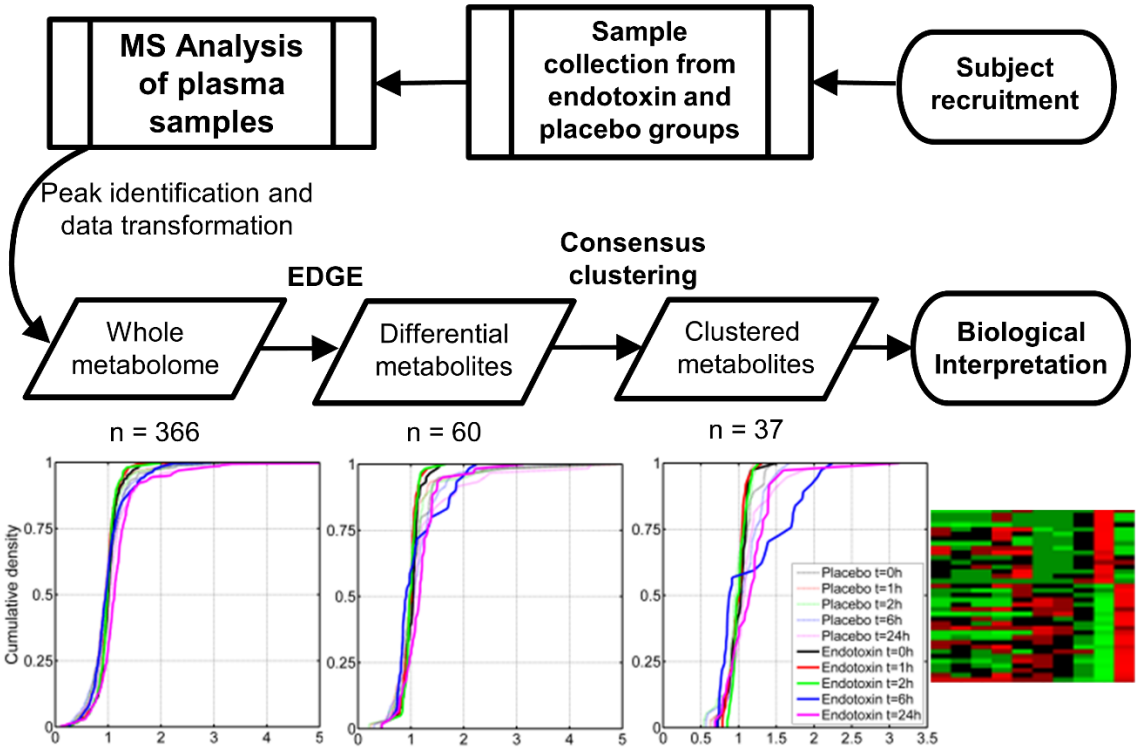

Figure 1: Study flowchart illustrating sample acquisition, biochemical profiling through MS, and data analysis steps. Diagrams below each data symbol display empirical cumulative distribution of the corresponding dataset, with the number of elements indicated below the data symbols.

The metabolite profiles obtained via biochemical profiling analysis contained information on 366 metabolites, including amino acids, carbohydrates, lipids, short peptides, nucleotides, cofactors and vitamins, and other various metabolites. As the aim of this study was not to examine changes in individual metabolites, but rather to identify the major dynamics of the human plasma metabolome as a whole, the data was filtered using an algorithm originally designed to be used in experiments involving gene microarrays. EDGE operates using a procedure that takes relevant information from every element in the dataset, in order to test each element to see if it is differentially expressed (Leek, et al. 2006). The application of this algorithm to the metabolic profile dataset allowed for identification of 60 metabolites that had differential temporal profiles between the LPS and saline groups (p-value < 0.05 and q-value < 0.1). The usefulness of this filtering algorithm in identifying metabolites whose profiles differ between the LPS and control groups can be seen in the change in cumulative distribution data prior to and following the use of EDGE (Figure 1). When the complete metabolome dataset is used, both treatment groups have nearly uniform distributions, but when only the differential metabolites are considered, the LPS group becomes distinguishable from the control group at certain time points in the study.

Following the identification of differential metabolites, PCA was used to determine the dominant patterns in the temporal profiles of these metabolites. The majority of the variance was captured by the first two principal components (PC1 and PC2), with PC1 capturing just over half of the variance (63%), as seen in Figure 2.d. The averages of PC1 and PC2 were plotted against time, and also against each other, for each of the two treatment groups (Figure 2.a-c). On average, there was a clear separation in both PC1 and

PC2 of subjects who had been treated with LPS, with control subjects showing less variation in PC2, and even less variation in PC1 than the LPS group. One way ANOVA was used on average PC1 values, and it was discovered that variation of PC1 over time for LPS groups is significant (p-value = 1.38×10^{-37}) whereas for the control group it is not ($p > 0.01$). Analysis of PC1 using Wilcoxon rank sum test analysis at each time point found that the most significant difference between the LPS and control groups occurred at the 6 hr time point (p-value = 0.00065), which was the time point that separated the development and recovery phases of the metabolic changes effected by LPS administration. Even at the 24 hr time point, average PC1 remained significantly differently between the groups (Figure 2.a), which indicates that the recovery phase was still in progress at that point.

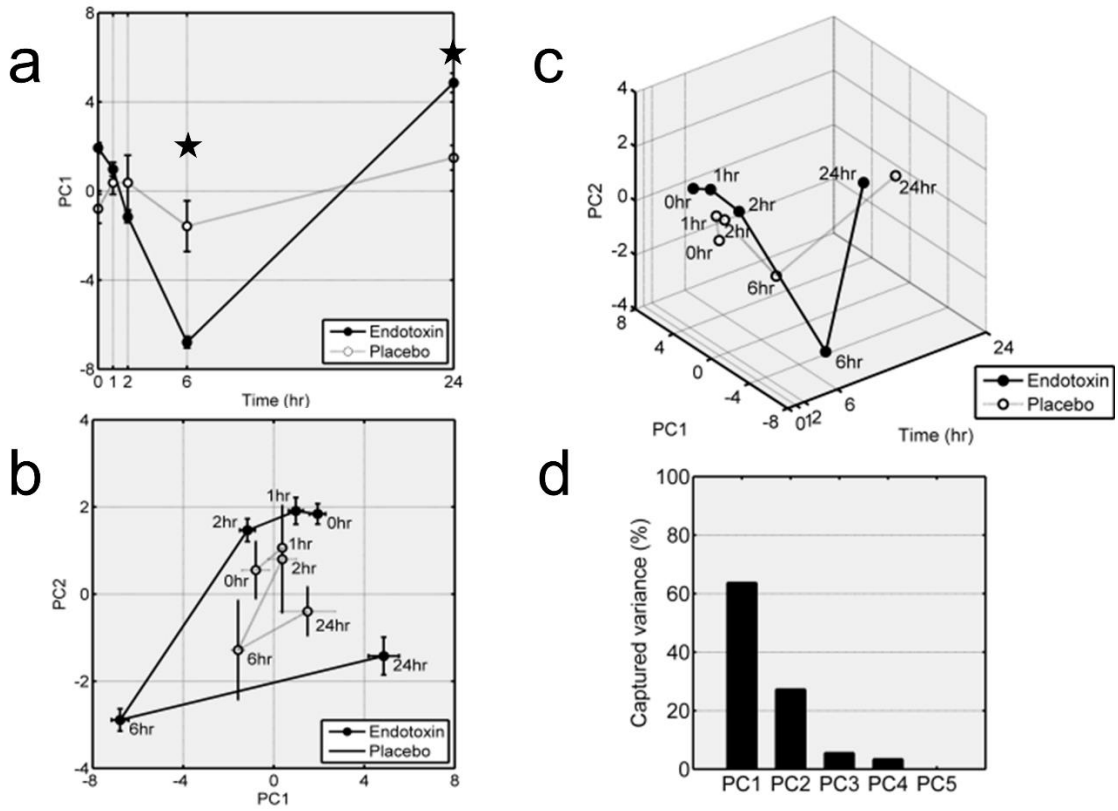

Figure 2: Principal component analysis. (a) Temporal changes in averaged PC1 for LPS and placebo treated subjects. (b and c) Trajectory averages in PC1-PC2 coordinates (b) and time-PC1-PC2 space (c). (Star sign indicates significance ($p < 0.01$) measured by Wilcoxon rank sum test and error bars indicate standard error of the mean).

Next, consensus clustering was performed on the metabolites that had previously been identified as having differential temporal profiles, in order to find subsets of metabolites that exhibited common response profiles (Nguyen, Nowakowski and Androulakis 2009). Consensus clustering is an important tool in looking at large datasets, as it allows for identification of coherent patterns of change over time, and enables a focus on elements of the data set that appear to have associated interactions. Along with this, consensus clustering also aids in identifying temporal relationships between subsets of the elements of the dataset, which could imply some sort of regulatory hierarchy (Nguyen, Nowakowski and Androulakis 2009).

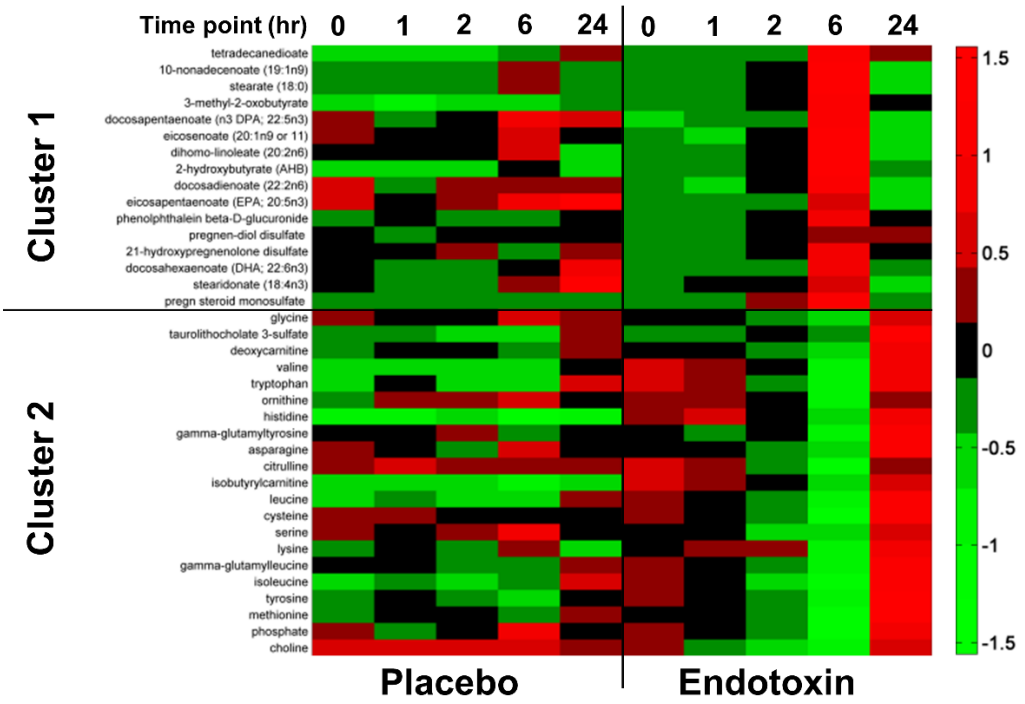

Figure 3: Heat map displaying the differential patterns of metabolic response to LPS. Two clusters of plasma metabolites reflect two distinct patterns with opposing temporal directionality. Clustered metabolites and their associations with the metabolic pathways are also listed in Table 1.

Table 1: Distribution and classification of the differential metabolites to the clusters shown in Figure 3.

	BIOCHEMICAL	SUB-PATHWAY	SUPER PATHWAY
Cluster 1	2-hydroxybutyrate (AHB)	Cysteine, methionine, SAM, taurine metabolism	Amino acid
	3-methyl-2-oxobutyrate	Valine, leucine and isoleucine metabolism	
	Docosahexaenoate (DHA; 22:6n3)	Essential fatty acid	Lipid
	Docosapentaenoate (DPA; 22:5n3)		
	Eicosapentaenoate (EPA; 20:5n3)		
	Tetradecanedioate	Fatty acid, dicarboxylate	
	Stearidonate (18:4n3)	Long chain fatty acid	
	Dihomo-linoleate (20:2n6)		
	Docosadienoate (22:2n6)		
	10-nonadecenoate (19:1n9)		
	Eicosenoate (20:1n9 or 11)		
	Stearate (18:0)		
	21-hydroxypregnenolone disulfate	Sterol/Steroid	
	Pregn steroid monosulfate		
	Pregnen-diol disulfate		
	Phenolphthalein beta-D-glucuronide	Detoxification metabolism	Xenobiotics
Cluster 2	Asparagine	Alanine and aspartate metabolism	Amino acid
	Cysteine	Cysteine, methionine, SAM, taurine metabolism	
	Methionine		
	Glycine	Glycine, serine and threonine metabolism	
	Serine		
	Histidine	Histidine metabolism	
	Lysine	Lysine metabolism	
	Tyrosine	Phenylalanine & tyrosine metabolism	
	Tryptophan	Tryptophan metabolism	
	Citrulline	Urea cycle; arginine, proline metabolism	
	Ornithine		
	Isobutyrylcarnitine	Valine, leucine and isoleucine metabolism	
	Isoleucine		
	Leucine		
	Valine		
	Phosphate	Oxidative phosphorylation	Energy
	Taurolithocholate 3-sulfate	Bile acid metabolism	Lipid
	Deoxycarnitine	Carnitine metabolism	
	Choline	Glycerolipid metabolism	
	Gamma-glutamylleucine	Gamma-glutamyl	Peptide
	Gamma-glutamyltyrosine		

After consensus clustering, the dataset was narrowed to 37 out of the 60 differential metabolites, which were grouped into two different clusters showing opposing temporal directionalities (Figure 3). A list of the metabolites found in each cluster and the metabolic pathways they are known to be associated with can be seen in Table 1. Cluster 1, which contained 16 metabolites, was up-regulated during the first 6 hr of the study, and down-regulated by the 24 hr time point. This cluster consisted mainly of metabolites that are associated with pathways related to lipid metabolism. Cluster 2, which contained 21 metabolites, showed an opposite directionality, and was down-regulated through the first 6 hr following LPS, but up-regulated at the 24 hr time point. Out of this cluster, 14 of the metabolites were amino acids or amino acid derivatives, and another 2 were dipeptides, which indicates that this cluster was related to a significant shift in protein metabolism.

DISCUSSION

This study used untargeted analytical methods and unsupervised data analysis to examine plasma metabolite levels following low dose LPS exposure, and to identify coherent patterns in the temporal changes of these metabolites. The most noticeable differences between the LPS and control groups were found in the levels of lipids, and of amino acids, which exhibited opposing directionalities in two distinct clusters. Plasma lipid levels steadily increased during the 6 hr following the administration of a low dose of LPS, before reversing direction during the recovery phase. In contrast, plasma levels of amino acids and amino acid derivatives decreased in the first 6 hr after LPS, before also reversing direction during the recovery phase.

In the first cluster of metabolites, which showed an increase in plasma levels, followed by a decrease, 13 out of 16 metabolites were lipids. More specifically, these were both

essential and non-essential long chain fatty acids (FAs), including omega-3 FAs, omega-6 FAs, and a major saturated FA, stearate. This up-regulation in plasma FA levels at the 6 hr time point is consistent with lipolysis, which is a well-known response to inflammation (Fong, et al. 1990). While the mobilization of lipid stores as free FAs in the plasma was initially thought to be a direct result of catecholamine release in response to injury, the signaling interactions which cause this lipolysis are now known to be much more complicated, and an investigation of the direct and indirect effects of various factors on lipid homeostasis requires more research (Glass and Olefsky 2012).

This cluster also contained 2-hydroxybutyrate (or α-hydroxybutyrate; AHB), which is a by-product of the pathway for glutathione synthesis from methionine. Glutathione is a vital antioxidant protein, and plays a very important role in mitigating oxidative damage caused by reactive oxygen species, whose formation can potentially be caused by inflammation (Jaeschke 2011). Combined with a decrease in levels of other factors involved in the glutathione synthesis pathway, an increase in AHB levels is indicative of an increase in hepatic oxidative defense mechanism activity, in order to combat oxidative stress brought on by LPS administration.

The second cluster contained metabolites whose concentrations decreased until 6 hr following the administration of LPS, before returning to normal by the 24 time point. Of the 21 metabolites in this cluster, 14 were amino acids, including 12 proteinogenic amino acids, and 2 important members of the urea cycle. These urea cycle members, combined with the presence of 2 intermediates in the amino acid degradation pathway, indicate that the amino acids are being used as substrates in energy production, along with being used in the liver to produce acute phase proteins.

Also in this cluster was Taurolithocholic acid 3-sulfate, which is produced by bile acid sulfation. Under normal conditions this is a minor pathway, but in the presence of intrahepatic cholestasis, which is known to be associated with inflammation (Khovidhunkit, et al. 2004), this reaction is enhanced, increasing the renal clearance of such compounds (St-Pierre, et al. 2001). The increase in plasma concentration of sulfate bile acids could indicate that LPS-induced inflammation resulted in a decrease in renal function. Along with this, increased levels of phosphates have been identified as a possible risk factor that may be linked to renal failure (Voormolen, et al. 2007). The fact that these independent markers of a decrease in renal function both appear in the second cluster, and share similar temporal patterns, suggests that LPS treated subjects may experience a decline in renal function.

EFFECTS OF COUPLED DOSE AND RHYTHM MANIPULATION OF PLASMA CORTISOL LEVELS ON LEUKOCYTE TRANSCRIPTIONAL RESPONSE TO ENDOTOXIN CHALLENGE IN HUMANS

Many immune system parameters undergo rhythmic fluctuations over the course of each day, controlled by autonomic and endocrine factors. The interaction between the immune and neuroendocrine systems also works in the opposite direction, with immune effectors, especially cytokines, influencing the autonomic and endocrine rhythms (Mavroudis, et al. 2012). When the body experiences trauma, infection, or other acute stress, the resulting inflammatory response is a coordinated action of both the immune and neuroendocrine systems, mediated in a large part by the hypothalamus-pituitary-adrenal (HPA) axis. The HPA axis controls circadian and ultradian glucocorticoid (GC) secretion rhythms, which are considered to be essential for the establishment of a basis for "continuous dynamic equilibriation", which is vital for achieving homeostasis and allostasis (de Kloet and Sarabdjitsingh 2008) (Lightman and Conway-Campbell 2010).

One of the major circulating human GCs involved is cortisol, which works through both feedback and feedforward loops to regulate this dynamic state and allow the body to respond properly to stressors. Ultradian oscillations of GC concentration have been shown to be critical for maintaining homeostatic balance in the body during downstream responses through mathematical modeling of the regulatory mechanisms. Mean GC responsive mRNA synthesis was determined to be greater with decreased pulsatility in in plasma cortisol levels, and other factors such as the concentration and timing with respect to the stressor also affected the stress response (Scheff, Calvano, et al. 2012). This combination of parameter effects contributes to a wide variation of inter-individual reactions to similar stressors (Young, Abelson and Lightman 2004).

GCs are primarily involved in stress response through immunosuppressive and anti-inflammatory actions, which have been used for decades in clinical treatment of autoimmune and inflammatory disorders. However, even in early views of GC physiology, there was the suggestion that rather than simply limiting the body's response to external stressors, endogenous GCs might actually be involved in enhancing and mediating this response. This view has been expanded to include new discoveries regarding the anti-inflammatory actions of GCs (Munck and Guyre 1986), and a view which accepted both the suppressive and permissive effects of GCs emerged. Under this view, GCs are seen as being able to permit or suppress the response to stressors, as well as being able to prepare or stimulate the response, depending on factors such as the timing, concentration, and duration of GC exposure. The only GC action linked to basal GC levels is the permission of the response, while the other actions are primarily observed in situations where the GC concentration has increased above basal levels. However, permissive action has also been associated with higher than basal levels of concentration when this occurs prior to an acute stressor (Sapolsky, Romero and Munck 2000).

Because of the major role that GCs play in regulating stress response, and because they are widely used in pharmacological treatment of many inflammatory diseases, it is important to fully understand their effects on immune cells. Previous studies in humans have looked at the effects of variations in GC dosage in response to lipopolysaccharide (LPS) challenge, and have established the role of GCs in permitting and stimulating immune response (Barber, et al. 1993) (Yeager, Rassias, et al. 2009). However, the endpoints of these experiments have been limited to measurements involving circulating

cytokines. Given the complexity of the immune system, studies focusing on single immunological parameters are not sufficient. Instead, system-wide approaches like global transcriptional profiling can be used to provide a broader scope for investigating physiological alterations that occur in response to stimuli, allowing for a more complete and better definition and understanding of complex biological phenomena (Ricciardi-Castagnoli and Granucci 2002). Previously, this approach has only been used to profile the transcriptional responses of blood cells that were stimulated by GCs *ex vivo* (Galon, et al. 2002) (Ehrchen, et al. 2007).

One GC-related condition is hypercortisolemia, which can cause major physical and psychological traumas. It is the result of both a disruption of the normal rhythm of cortisol secretion and an increase in total cortisol concentration (Deuschle, et al. 1997) (Vaughan, et al. 1982). An understanding of how immune function is affected by hypercortisolemia could have major clinical impacts, especially for critically ill patients, if it could be used to prevent conditions such as sepsis from occurring.

By looking at transcriptional profiling of whole blood leukocytes *in vivo*, this study assessed their response to coupled rhythm and dose manipulation of cortisol levels preceding an immune challenge caused by the administration of LPS. Healthy human model LPS administration has been shown to be an effective and reproducible platform for investigating the mechanism of cell and organ response to systemic inflammation, with many physiologic and metabolic processes being altered in a similar manner to the changes observed following acute injury and systemic inflammation (Lowry 2004). Current study design includes mimicking hypercortisolemia by blunting the natural circadian rhythm in cortisol fluctuation via a continuous cortisol infusion over a period of

30 hours, at a concentration higher than the peak of endogenous levels. At the 24 hr mark during this infusion, a bolus injection of LPS is administered, to present an immune challenge. The transcriptional responses of leukocytes were determined for 24 hr periods before and after the LPS administration, using gene expression analysis.

METHODS

Human subjects and sample collection:

Nine healthy volunteers were found, meeting a specific set of criteria, including age (between 18 and 40), and normal health. Subjects provided consent, and underwent initial screening to ascertain their suitability for inclusion in the study. Those who were determined to be eligible for the study were admitted to the clinical research center within three weeks.

Subjects were randomized, and received either intravenous saline infusion (n=4) or a cortisol infusion of 3 mcg/kg (n=5) for 30 hr, which began 24 hr prior to the administration of the endotoxin. At 0900 clock time, which was designated the zero hour time point in the study, subjects were given a bolus dose of 2 ng/kg of endotoxin (CC-RE, lot 2), which was administered intravenously, as previously described (Lowry 2004) (Haimovich, et al. 2010). At various time points both prior to and following endotoxin administration, blood samples were taken and vital signs were recorded. During the study, subjects avoided physical activity other than walking, and were attended to by nurses around the clock.

Blood plasma from the subjects was analyzed via radioimmunoassay to determine blood plasma cortisol concentration, as previously described (Hawes, et al. 1993). Using standard laboratory procedures carried out at the Robert Wood Johnson University

Hospital clinical pathology laboratories, whole blood levels of total leukocytes were determined for the various samples. These samples were taken and the leukocytes were isolated at various time points throughout a 48 hr period (Figure 4), starting when the cortisol or saline infusion began, and ending 24 hr after the administration of the endotoxin. After RNA extraction and cRNA synthesis, total cellular RNA is hybridized onto the GeneChip® Human Genome Focus Array (Affymetrix), as previously described (Calvano, et al. 2005).

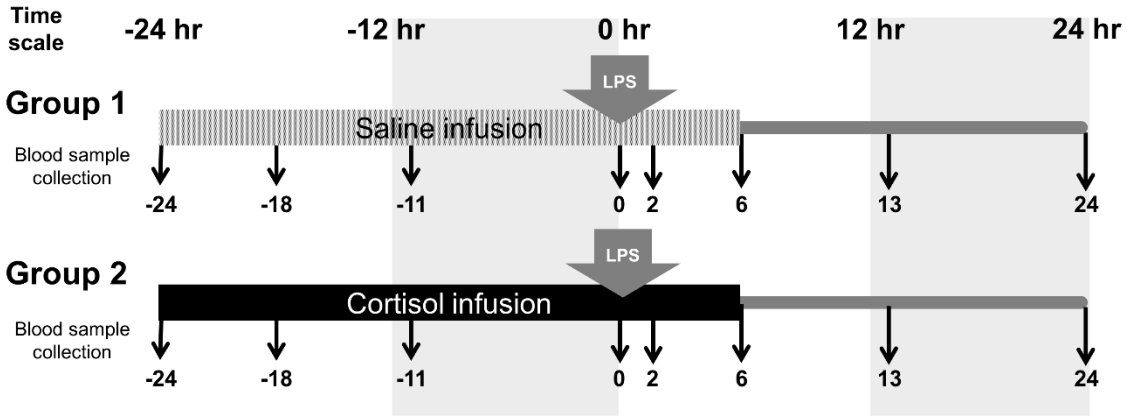

Figure 4: Study design. Nine healthy subjects received continuous cortisol (or saline) infusion starting 24 hr before the bolus intravenous endotoxin (LPS) injection and continuing until 6 hr after the injection. Blood samples were collected and leukocytes were isolated at multiple time points over 24 hr both before and after LPS administration.

Microarray data analysis:

In this study, data analysis included extraction of expression values, identification of treatment group members, filtering for probesets of interest, and clustering. DNA chip analyzer (dChip) software was used to generate expression values from the microarray data, using invariant-set normalization and perfect match modeling. Probesets of interest were identified using software for the extraction and analysis of gene expression (EDGE) (Leek, et al. 2006). EDGE was used to determine probesets differentially expressed over

time for all subjects. Principal component analysis (PCA) was then performed using these probesets, and the first principal component (PC1) was plotted vs. time for each subject (McDunn, et al. 2008). The resulting projections were clustered in MATLAB using wavelet-model based clustering to identify the members of the two treatment groups.

EDGE was then used to identify probesets differentially expressed between the two treatment groups. Finally, the filtered data sets were clustered using "consensus clustering", to identify subsets of the probesets with common coherent expression patterns (Nguyen, Nowakowski and Androulakis 2009). Functional annotation of these probesets was performed via the pathway enrichment function in Ingenuity Pathway Analysis tools (IPA, Ingenuity® Systems, www.ingenuity.com), using the databases of IPA and Kyoto Encyclopedia of Genes and Genomes (KEGG) to analyze the function of each of the genes that corresponded to the probesets identified by the consensus clustering.

Promoter analysis:

Genomatrix, a database containing promoter information, was used to identify promoters of genes associated with the clusters obtained by analysis of the time period from -24 to 0 hr. Cortisol responsive genes were identified by looking for the presence of glucocorticoid response elements (GREs) on gene promoters. Regions that were conserved across the identified promoters of the selected genes were located, and the GRE binding site motif (TGTTCT (Almon, et al. 2005)) was compared against the conserved regions, to find any matches (Nguyen, Almon, et al. 2010). This was done for

all genes associated with the clusters from the time period prior to endotoxin administration (-24 hr to 0 hr).

RESULTS

The aim of this study was to examine the effects of combined dose and rhythm manipulation of plasma cortisol levels on the transcriptional changes in humans caused by acute LPS exposure. The study involved two groups of subjects who were given either a saline or cortisol infusion over a 30 hr time period, with both groups receiving a bolus dose of LPS 24 hr following the start of the cortisol or saline infusion (Figure 4). Blood samples were taken at various time points, and microarray analysis was used to determine the transcriptional response of leukocytes. Plasma cortisol concentration and blood cell count were also measured.

Plasma cortisol levels and leukocyte count:

Before beginning saline or cortisol infusion, the baseline plasma cortisol concentrations for both the saline+LPS and cortisol+LPS groups were 10-15 μg/dL. At the -24 hr time point, the cortisol+LPS group began receiving the cortisol infusion, and their plasma cortisol levels increased to ~35 μg/dL. In the saline+LPS, plasma cortisol levels varied between 2 and 15 μg/dL, which was expected due to the natural circadian rhythm (Figure 5). At the 0 hr time point, LPS was administered, and while plasma cortisol levels remained fairly steady for the cortisol+LPS group, there was a steep increase in cortisol concentration within the first 3 hr for the saline+LPS group. Plasma cortisol concentrations for this group increased from around 10 up to 23 μg/dL during this 3 hr time period after LPS administration, before beginning to return towards the baseline. For the cortisol+LPS group in the first 6 hr following LPS administration, plasma cortisol

levels stayed close to 30-35 μg/dL. At this point, the cortisol infusion was ended, and cortisol levels for this group returned to normal in the following hours.

Directly prior to administration of LPS, a significant difference was observed in the total number of leukocytes between the saline treated group and the cortisol treated group. The continuous infusion of cortisol caused a significant increase in the total number of white blood cells (WBCs), to a level only observed in the saline treated group following LPS administration. The number of WBCs continued to increase for the cortisol+LPS group after LPS administration, at a rate comparable to that of the saline+LPS group (Figure 5). Also, the leukocyte subpopulations, when measured as a percentage of the total number of leukocytes, were not significantly different by 3 hr following the administration of LPS.

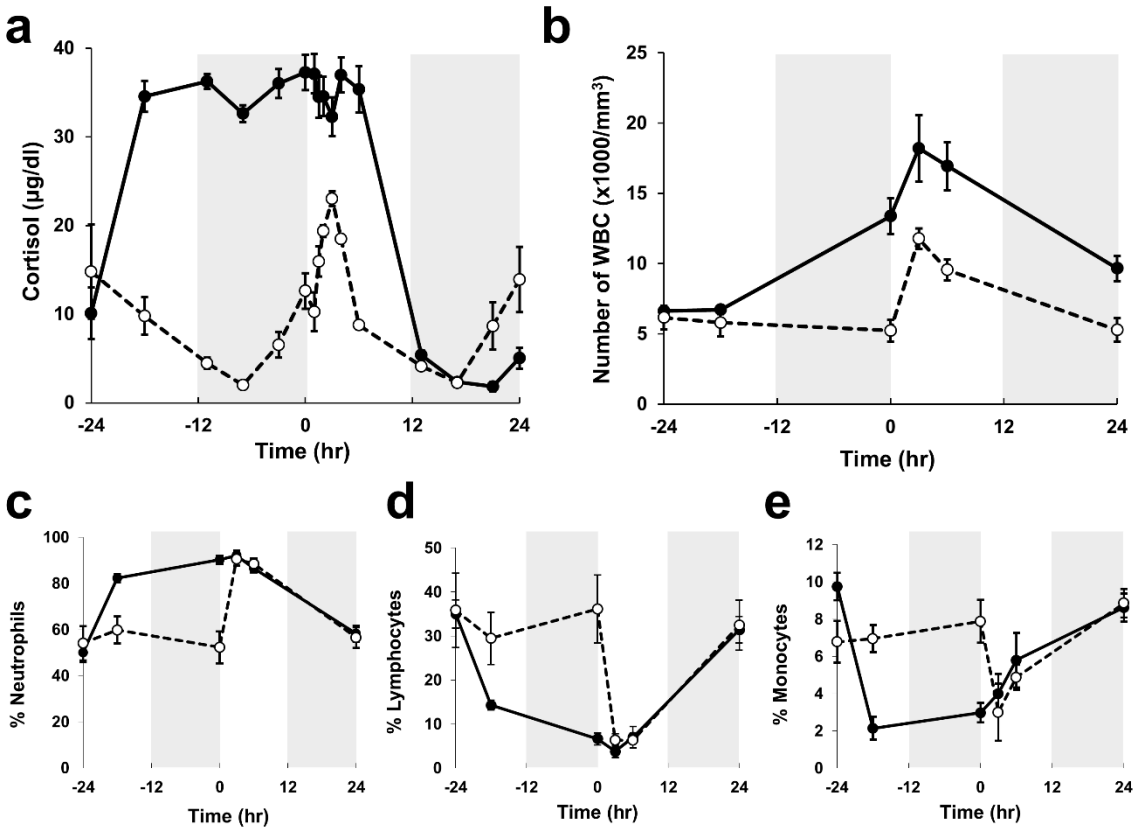

Figure 5: Change in cortisol and WBC levels. Change in the level of plasma cortisol (a) and total number of white blood cells (WBCs) (b) and percentage of WBC subpopulations (c-e) in response to continuous cortisol infusion and LPS administration (dashed line: saline + LPS; solid line: cortisol + LPS).

Identification of pre-treatment groups from gene expression data:

In the initial examination of the microarray data, each subject was assigned into one of the two treatment groups based solely on the gene expression patterns, with actual assignments of subjects being kept anonymous. For this purpose, the probesets which were differentially expressed over time for all subjects were identified ($p<0.01$, $q<0.01$), resulting in 799 probesets of interest out of 8793 total. Then, principal component analysis (PCA) was performed on the differentially expressed probesets. The first principal component (PC1), which accounted for ~50% of the total variance, was plotted over time for each of the nine subjects and resulting profiles were then clustered.

Averaged PC1 profiles of two resulting clusters are shown in Figure 6. The final assignment according to these clusters placed four subjects in one group (saline pre-treated, dashed line), and five in the other (cortisol pre-treated, solid line). This assignment of subjects was verified by the physicians who performed the study.

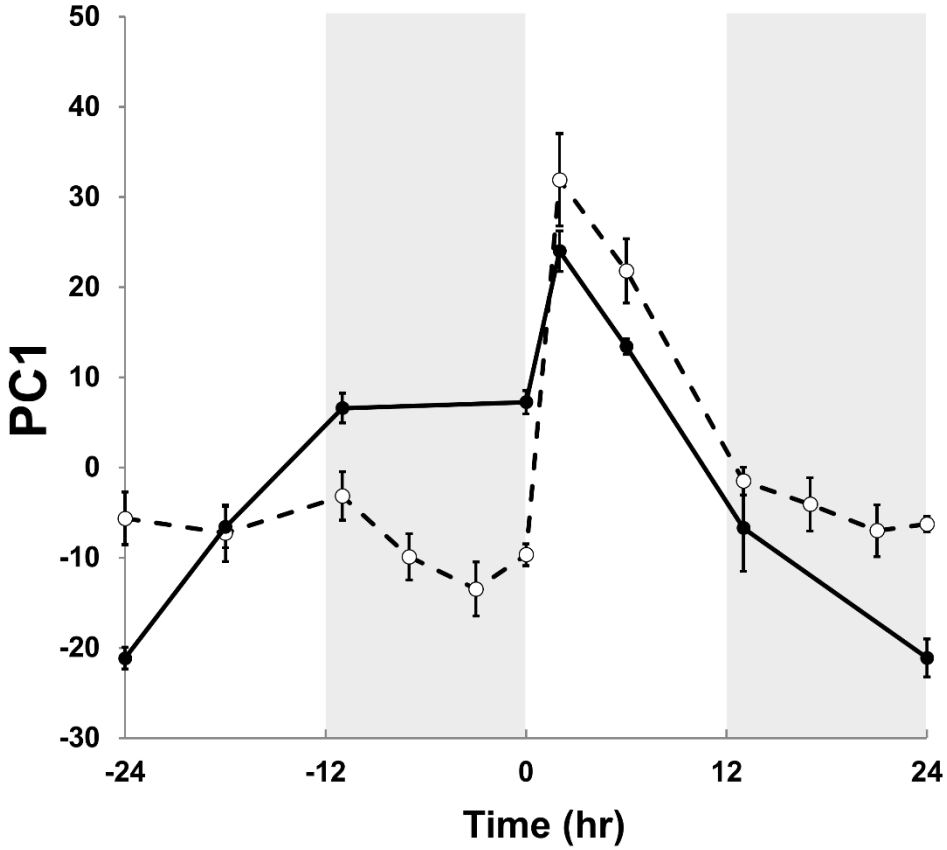

Figure 6: Principal component groupings. Averaged first principal components (PC1) for saline (dashed line) and cortisol (solid line) treated groups.

Transcriptional effects of manipulated plasma cortisol levels:

Following the initial examination of the transcription data, the scope of the analysis was narrowed to focus on the first 24 hr of the study, during which the response to the cortisol infusion could be observed independently of the response to LPS exposure. Out of the time period from -24 hr to 0 hr, 199 out of a total of 8793 probesets were identified as being differentially expressed between the cortisol treated subjects and the saline control

group ($p<0.05$ and $q<0.05$). Using consensus clustering analysis, these probesets were separated into 6 different clusters, based on their temporal expression profiles (Nguyen, Almon, et al. 2010). These clusters were examined to identify which showed the cortisol group as up-regulated as compared to the saline group, and which showed down-regulation in the cortisol group when compared to the saline group. The resulting groupings can be seen in Figure 7.

Compared to the saline control group, 120 probesets were up-regulated and 80 probesets were down-regulated in the cortisol group. Representative genes from the clusters of up- and down-regulated genes are listed in Table 2, along with the functional groups associated with them. Examination of the temporal transcriptional profiles and associated functions of the probesets that are up- or down-regulated in the cortisol group reveals an increasing sensitization in response to the continuous infusion of cortisol. Many cytokine and pattern recognition receptors are up-regulated, along with signal transduction proteins and receptor regulatory elements. Mitochondrial complex elements and protein translation machinery are down-regulated, while two genes (UB2B, UBE2D1) that encode for enzymes that degrade proteins were up-regulated, which indicates a catabolic effect on overall levels of cellular protein. Cytoskeletal proteins are up-regulated, which could increase motility and enhance phagocytosis. Along with this, extracellular matrix degradation enzymes are up-regulated, indicating that the leukocytes are preparing to extravasate and move to the area of a possible infection.

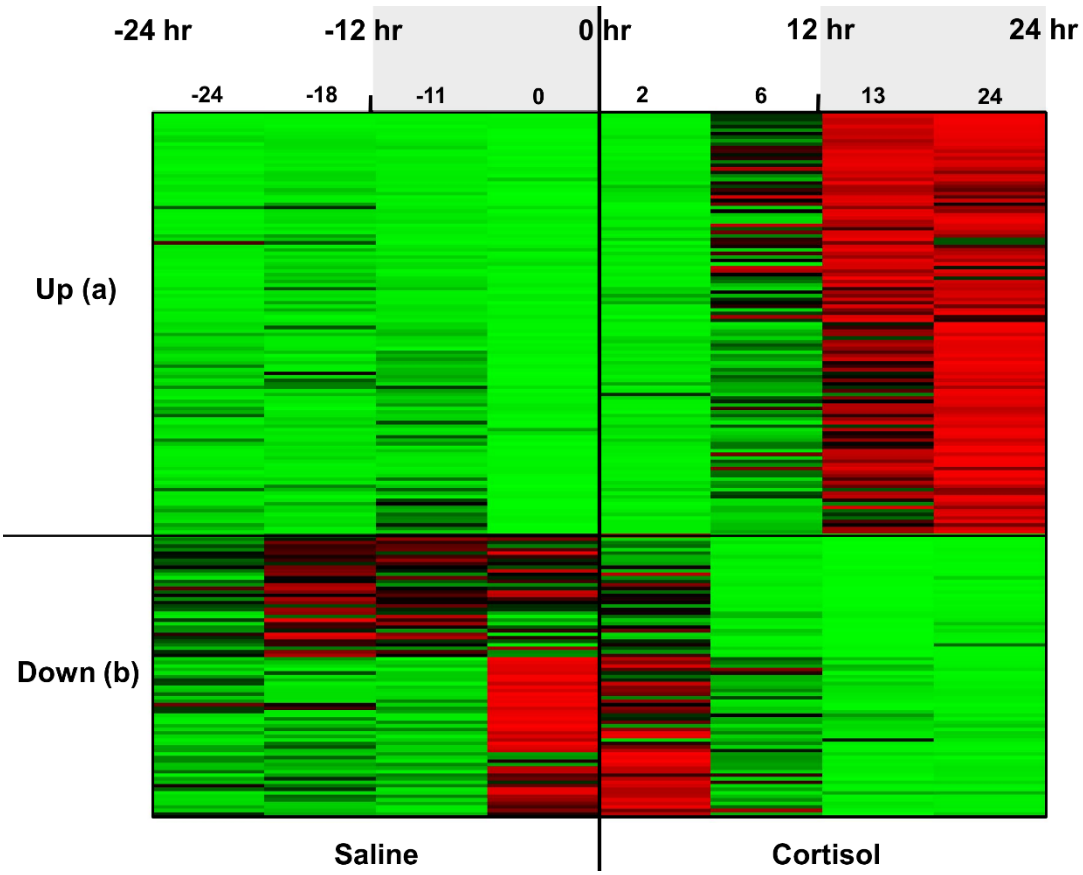

Figure 7: Transcriptional responses of leukocytes to cortisol prior to endotoxin administration in time period between -24 hr to 0 hr.

Table 2: Functional annotation and representative probesets associated with up- and down-regulated clusters in response to continuous cortisol infusion in -24hr to 0hr time period.

	Classification	Molecules
Up (a)	Cytokine and pattern recognition receptors	CSF2RA, IL10RB, IL13RA1, IL1R2, IL4R, TNFRSF10C, TNFRSF1A, TNFRSF9, TLR1, TLR8
	Receptor regulatory elements	IL18RAP, GRB10, IRS2
	Signal transduction proteins	NFKBIA, CAMK1D, LIMK2, MAP2K4, MAP2K6, PPP2R5A
	Transcription factors	ATF6, FOS, STAT5B
	Negative regulators of complement system	CD55, CD59, SERPINB1
	Cytoskeletal proteins	IQGAP1, GIT2, TUBA1A
	Protein degradation enzymes	UB2B, UBE2D1
	ECM degradation enzymes and their inhibitor	MMP25, MMP9, TIMP2
	Amino acid degradation	ARG1
Down (b)	Protein translation regulatory elements and translation machinery	EEF2, EIF3H, CCNC, RPSs – 5 probesets–, RPLs – 8 probesets–
	Mitochondrial proteins	NDUFs–3 probesets–, UQCRFS1
	Free radical scavenging	SOD1
	Transcription factors	LEF1, STAT4
	Antigen presentation	HLA-DPB1

Using the genes associated with the clusters identified in the time period from -24 hr to 0 hr, a promoter analysis was performed by searching for the GRE binding motif on conserved regions across sets of orthologous promoters. Out of 200 associated genes, 82 were identified as having a GRE-binding site, 50 genes had no GRE-binding site, and 68 genes were excluded because of limitations on promoter information. The genes that were found to have GRE-binding sites in their promoter regions did not appear to show a specific directionality in their regulation, with distribution of the genes into up- and down-regulated clusters having nearly equal weights.

Overall response to LPS:

Once the effects of cortisol on leukocyte transcription prior to LPS administration were examined, the analysis was expanded to include the full time period of the study, in order to examine how the response to LPS was affected by the continuous prior infusion of cortisol. Out of 8793 probesets, 199 were identified as being differentially expressed between the -24 hr and the 24 hr time points. 157 of these probesets were clustered into 5 clusters, based on their expression profiles. These clusters were then grouped and classified according to their response from -24 hr to 0 hr, as either LPS dominated, cortisol enhanced, or cortisol suppressed (Figure 8).

The heat maps of the probesets that were classified as cortisol enhanced and cortisol suppressed (Figure 8.b and 8.c) are consistent with the previous analysis of the time period from -24 hr to 0 hr, showing up-regulation and down-regulation, respectively, in response the continuous cortisol infusion. Response patterns following LPS administration were similar for both groups, across each of the clusters, but the magnitude of the up-regulation in response to LPS was greater in the cortisol group than in the saline control group. The cluster in Figure 8.a shows an expression profile where the response appears to be predominately driven by LPS, exhibiting a strong up-regulation in the first 2 hr following the LPS administration, which was again greater for the cortisol group than for the control group.

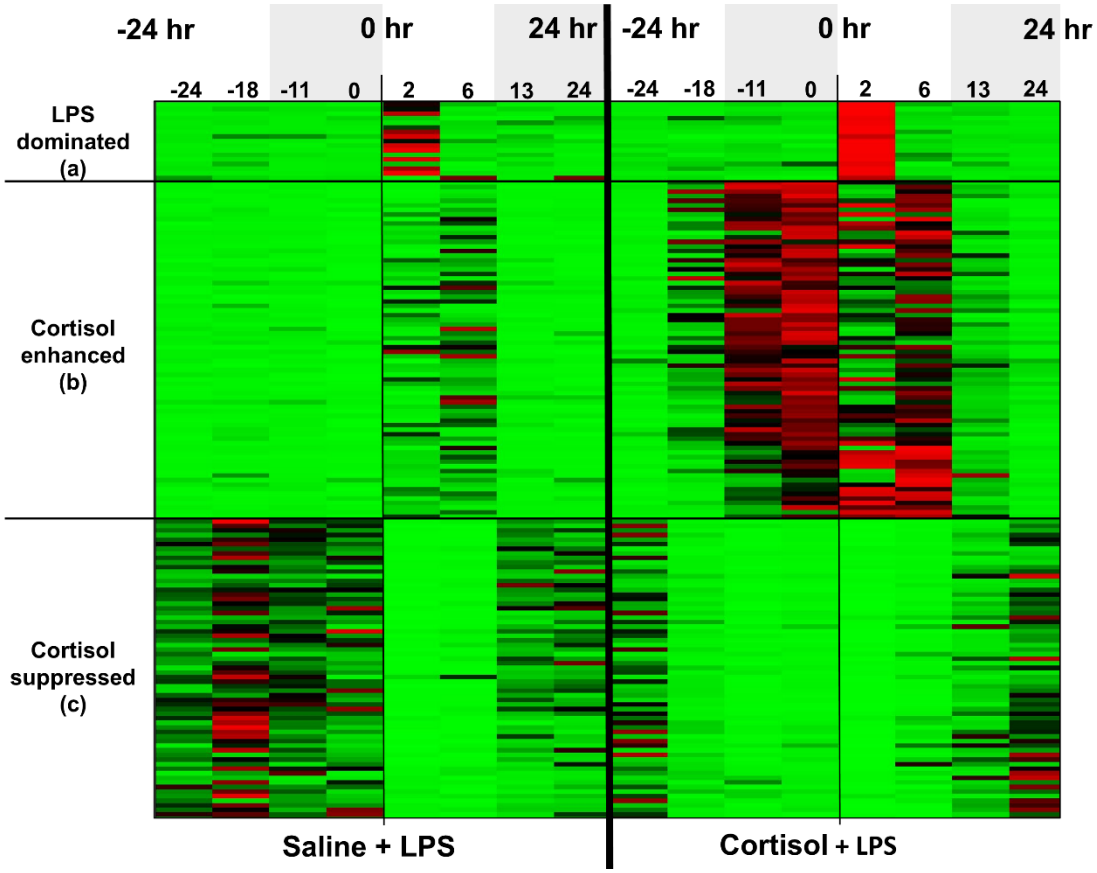

Figure 8: Overall differential response to LPS between cortisol+LPS and saline+LPS groups. 5 clusters obtained by consensus clustering were grouped into 3 based on response to cortisol within -24 hr to 0 hr as: (a) LPS dominated (17 probesets), (b) cortisol enhanced (74 probesets), and (c) cortisol suppressed (66 probesets).

DISCUSSION

The design of this study involved using continuous infusion of cortisol over the course of 30 hr, at a concentration high enough to raise plasma cortisol levels to a level generally associated with major physical stress (Yeager, Pioli and Guyre 2011), in order to blunt the natural circadian rhythm in healthy volunteer subjects. A bolus injection of LPS was administered 24 hr after the start of the continuous cortisol infusion. The transcriptional response of leukocytes was analyzed for the cortisol infusion period in combination with the whole time period of 48 hr.

In control subjects, plasma cortisol concentrations exhibited the expected circadian patterns prior to LPS administration (Yeager, Pioli and Guyre 2011), and increased rapidly after the bolus injection of LPS. In subjects given the continuous cortisol infusion, plasma cortisol levels held fairly steady during the entirety of the 30 hr infusion period, at a level close to twice the normal peak level of the control subjects. No further increase was observed in cortisol levels following LPS administration for these subjects. For both groups, cortisol levels returned to normal concentrations within 12 hr of the LPS bolus injection. The pattern observed here indicates that the continuous cortisol infusion was sufficient to blunt the natural circadian rhythm of plasma cortisol levels and to maintain the cortisol concentration at a level normally associated with physiological conditions brought on by physical stress.

For the subjects treated with continuous cortisol infusion, the number of leukocytes present was significantly increased even prior to LPS administration, and increased even more after the LPS bolus injection, to an extent that was comparable to the increase seen in the saline control group. This increase in leukocytes contrasts the results of previous stress related studies in rats, which showed an inverse relationship between plasma corticosterone concentrations and the number of leukocytes present (Dhabhar, et al. 1995) (Dhabar, et al. 1994). One reason for this difference in results could be due to the method of affecting GC levels in the body. For most animal studies, corticosterone levels are increased by subjecting the animals to restraint stress over a short period of time (1-2 hr), while in this study, cortisol levels were fixed at a concentration associated with stress by infusion of cortisol over a period of 30 hr. Previous studies have shown that along with concentration, the timing, pulsatility, and duration of cortisol are also important in

determining the effect on various physiological processes (Sapolsky, Romero and Munck 2000), so this difference in study designs could be the reason for observing contradictory results in the number of leukocytes.

Observation of the transcriptional response over the initial 24 hr of the study allowed for understanding of the changes in regulation of leukocytes when exposed to consistently high levels of plasma cortisol concentration. These changes included an increase in the expression of genes coding for several cytokine and pattern recognition receptors, signal transduction elements, and receptor regulatory elements. Along with this, there was a decrease in the expression of genes that code for elements involved in protein translation, and for mitochondrial proteins. The increase in receptor and signal transduction protein expression indicated that the higher cortisol concentration served to prime the immune response, sensitizing cells to recognize and respond to potential infection or danger signals. This observation fits well with the results of previous studies on the effects of cortisol on the immune response, which showed increased pro-inflammatory receptor expression and function (Shieh, Peterson and Moore 1993) (Hawrylowicz, Guida and Paleolog 1994) (Zhang and Daynes 2007), resulting in an enhanced response to LPS (Lim, et al. 2007) (Frank, et al. 2010) (Yeager, Rassias, et al. 2009) (Galon, et al. 2002) (Johnson, et al. 2002) (Gundersen, et al. 2006).

This enhanced response is also aided by the up-regulation of cytoskeletal proteins and enzymes that degrade the extracellular matrix, allowing for increased motility and enhanced extravasation, in order to engulf pathogens or move through tissues. These functions are key to the effectiveness of leukocytes in the immune response, and the effect of cortisol in enhancing these functions supports its role in increasing the

effectiveness of the immune response, as seen in previous studies (Galon, et al. 2002) (van der Goes, et al. 2000).

The down-regulation of protein translation is also a part of the priming effect of cortisol, as it indicates a change in cellular energy and resource prioritization, which would allow a better response to an anticipated threat. A decrease in protein synthesis combined with an increase in the expression of enzymes which degrade proteins points toward a catabolic state, which is characteristic of cortisol as a stress hormone (Sapolsky, Romero and Munck 2000) (Shah, Kimball and Jefferson 2000) (Shah, Iniguez-Lluhi, et al. 2002). The overall effect of the cortisol infusion seems to be an increase in the preparedness of immune cells to respond to potential threats, creating a preparatory effect to enhance the effectiveness of the immune response. While this may appear to be counter-intuitive, since glucocorticoids are known for being anti-inflammatory and immunosuppressive, their effects depend heavily on concentration, duration, and timing of exposure, and cortisol has been shown to enhance and increase immune function under certain conditions (Sapolsky, Romero and Munck 2000) (Lim, et al. 2007) (Galon, et al. 2002) (Zhang and Daynes 2007).

The promoter analysis performed on the genes that were associated with differential expression patterns due to continuous cortisol infusion showed that less than half of those genes had a GRE-binding site in their promoter regions, meaning that glucocorticoid receptor (GR)-DNA binding was not directly responsible for the majority of the differential expression. However, this points to the previously known fact that GR also serves in a regulatory function through mechanisms other than direct DNA binding (Buckingham 2006) (Miranda, Morris and Hager) (Rao, et al. 2011). There was also no

indication of a preferred directionality in GR regulation, as genes with and without GRE-binding sites were found in both up-regulated and down-regulated clusters. This is not surprising, given the complexity of the mechanisms involved, and the wide variety of functional consequences of glucocorticoids (Sapolsky, Romero and Munck 2000) (Goulding 2004) (Busillo and Cidlowski 2013).

While there were significant transcriptional changes due to the cortisol infusion prior to the 0 hr time point and the LPS administration, the overall response to LPS was not significantly different, as seen in the clusters of the genes that were differentially expressed over the full 48 hr period. Both the saline control group and the cortisol group exhibited similar up- and down-regulation responses following LPS, as seen in Figure 8.b and c. However, the expression profile in Figure 8.a is very different. The increase in expression of genes in this cluster is very steep, and overrides any possible cortisol effect that may have been present prior to LPS. The response to LPS was much higher in the cortisol group as compared to the control group, and indicates that while cortisol did not directly affect the expression of genes in this cluster, it could have affected their regulation indirectly, and thus magnified their response to the administration of LPS. This cluster includes probesets that are associated with some of the major regulators of the inflammatory response, including receptors involved in leukocyte recruitment, cytokines and chemokines, and signal transduction elements involved in negatively regulating pro-inflammatory signaling. As a whole, these results indicate that continuous cortisol infusion serves to sensitize leukocytes and to prepare them to respond efficiently in the event of a potential infection.

CONCLUSION

Overall, in the first study, LPS administration in healthy volunteers was observed to cause significant alterations in lipid and protein metabolism homeostasis within the first 6 hr following LPS. By the 24 hr time point, these metabolite balances had moved back toward recovery. The changes in plasma lipid levels were likely associated with the known lipolytic effects of inflammation, while the early animo acid deficiency may have been caused by an increase in hepatic uptake, in order to meet an increased demand for substrates involved in acute phase protein synthesis and anti-oxidant defenses. Also, a later increase in markers that are associated with renal failure indicated that kidney function may have been affected in those subjects who were treated with LPS.

When considering the results of this study, an important point to note is that while the experimental model of human endotoxemia used in this study provides a good simulation of systemic inflammation, this simulated systemic inflammation is best described as being TLR4 agonist induced (Calvano and Coyle 2012). Subjects are screened to check for normal health prior to inclusion in the study, so it is important to take care in extrapolating the results of such a study to address conditions such as sepsis, which are clinically more complex. The data filtering and clustering techniques used in this study, while helping to identify common patterns, may inhibit the identification of subtler changes which do not conform to the dominant patterns. For example, metabolites such as lactate, which have an early perturbation that is quickly resolved, may be overlooked after filtering and clustering (Michaeli, et al. 2012). The limited number of time points in the study, and the large time gap between the last two time points, may also mask the

action of any metabolites that exhibit dynamics between those two time points, rather than at the 6 hr time point.

In the second study, the natural circadian fluctuation of plasma cortisol levels was blunted with the continuous infusion of cortisol, which was then followed by a bolus injection of LPS, to provide an immune challenge. Plasma cortisol levels, total leukocyte count, and leukocyte transcriptional response were monitored starting 24 hr prior to the administration of LPS, and ending 24 hr following LPS bolus injection. The plasma cortisol concentration was maintained around 35 μg/dL, at a level consistent with major physical stress.

This caused an increase in the total number of leukocytes, as well as altering their transcriptional response, creating a priming effect for immune response. The priming effect resulted from the increase in expression of cytokine and pattern recognition receptors, as well as signal transduction and receptor regulatory elements, along with a decrease in protein translation and mitochondrial proteins. Less than half of this priming effect was a direct result of GR-DNA binding, according to promoter analysis, so other transcription regulation mechanisms must have played a part in priming the leukocytes to respond.

However, these changes prior to LPS administration did not result in a significantly different overall response to LPS between the cortisol group and the saline control group, except for in one cluster, which contained probesets associated with some of the more important players in the inflammatory response. These included cytokines and chemokines, receptors involved in leukocyte recruitment, and signal transduction elements that are anti-inflammatory. While their transcription was not directly affected by

the cortisol infusion itself, it was indirectly involved in regulating their transcription following LPS administration. Overall, the continuous cortisol infusion resulted in a priming effect on the leukocytes in the blood, as seen in the sensitization of their surveillance processes, and the re-prioritization of energy distribution in the cells, to allow them to respond more efficiently to possible infectious threats.

In both studies, the use of untargeted bioinformatics-based exploration of the data allowed for the identification of the dominant patterns of response to the LPS exposure and, where applicable, to the continuous cortisol infusion. After clustering the plasma metabolite data, two clusters, one composed mainly of lipids and one composed mainly of amino acids, showed opposing directionalities following LPS exposure, with each cluster having a distinct temporal pattern. For the leukocyte transcriptional data, the identification and clustering of probesets differentially expressed over time was used to correctly identify both the saline control group and the cortisol group without any prior knowledge of group assignments, and then to separate out three types of response to LPS exposure – cortisol suppressed, cortisol enhanced, and LPS dominated. Clustering the probesets differentially expressed between the cortisol group and the saline control group prior to LPS exposure resulted in two clusters, one up-regulated and one down-regulated in response to cortisol, and examination of the functions associated with each cluster indicated a priming effect on the immune response. While metabolites or genes not conforming to the dominant expression patterns may need to be studied separately, the use of non-targeted and unsupervised data analysis and global transcriptomic studies allows for examination of the immune response as a whole, providing understanding of patterns of response beyond single immunological parameters.

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1. Kamisoglu K, **Sleight K**, Nguyen TT, Calvano SE, Coyle SM, Corbett SA, and Androulakis IP. 2014. "Effects of coupled dose and rhythm manipulation of plasma cortisol levels on leukocyte transcriptional response to endotoxin challenge in humans." *Innate Immunology* 20 (7): 774-84.

2. Kamisoglu K, **Sleight KE**, Calvano SE, Coyle SM, Corbett SA, and Androulakis IP. 2013. "Temporal metabolic profiling of plasma during endotoxemia in humans." *Shock* 40 (6): 519-26.