Acute Psychosocial Stress Induces Changes in Norepinephrine Sensitivity of Stimulated TNF-α Production

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ABSTRACT

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Background and Rationale: It is well established that psychosocial stress induces changes in glucocorticoid (GC) sensitivity, but little is known about how repeated psychosocial stress induces changes in norepinephrine sensitivity of stimulated TNF-α production. Psychosocial stress induces an increase in blood concentrations of catecholamines, which are important modulators of the immune system. Since stress is known to exacerbate the symptoms of autoimmune disorders, which disproportionally affect women, and catecholamine resistance has been reported among women with autoimmune disorders, it is important to study inflammatory cytokine sensitivity catecholamine differences in both men and women. Little is also known about how catecholamine responses to stress and catecholamine sensitivity of immune cells changes with increasing age. Thus, the current study set out to assess gender and age differences in alterations of *in vitro* sensitivity of inflammatory cytokine production to the effects of norepinephrine.

Methods: Thirty-five younger (18-35) and older (50-64) males (N=12) and females (23) were subjected to the Trier Social Stress Test (TSST) on two consecutive days, and salivary alpha-amylase responses and catecholamine sensitivity were measured. Enzyme-linked

immunosorbent assay (ELISA) kits were used to analyze concentrations of the pro-inflammatory cytokine Tumor Necrosis Factor α (TNF- α), and colorimetric assays were used to analyze alpha amylase concentrations.

Results: Men and women significantly differed in sensitivity on day 1 (time*gender effect: $F_{4, 148}$= 3.464*, p*< .01), where females showed a decrease in sensitivity and males show increased sensitivity. However, gender differences were not found on day 2 (time*gender effect: $F_{4, 176}$= 1.636*, p*< .167), where men and women both showed decreases in catecholamine sensitivity. Older (50-64 years) and younger (18-35 years) groups did not significantly differ in catecholamine sensitivity overall ($F_{5,165}$= 0.670*, p*= .647). Figure 3 and Table 1 could not be embedded within the file.

Conclusions: To the best of our knowledge, this is the first report of showing gender differences with inflammatory cytokine production to the immunosuppressive effects of catecholamines after repeated stress. Future studies on stress effects on inflammatory regulation will have to include assessments of both, glucocorticoid and catecholamine sensitivity in addition to measurements of circulating hormone levels.

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INTRODUCTION

Chronic low-grade peripheral inflammation is associated with an increased risk of developing atherosclerosis and subsequently cardiovascular disease (CVD) (Golia et al., 2014) which is the leading cause of death in the United States (WHO, 2014). Chronic low-grade inflammation can be the result of a wide variety of influences such as smoking, alcohol consumption, obesity, and chronic stress (Steptoe, Hamer, & Chida, 2007). Studying the inflammatory response is important since the mechanisms linking chronic stress and disease are poorly understood. Stress systems such as the sympathetic nervous system (SNS) are involved in stimulating stress-induced inflammation via the actions of catecholamines if the inflammatory response is quiescent. However, when introduced in the context of an ongoing inflammatory response, such as an infection, catecholamines actually inhibit the inflammatory response. It is well known that the effects of cortisol on the inflammatory system are variable since target cells are able to fine-tune their responses at all levels of the signal transduction cascade. Since the signal transduction cascade of catecholamines is just as complex as that of GCs, it may be possible that there is variation in the catecholamine effects on inflammation. In fact, one recent study found reduced sensitivity of TNF-α but not IL-6 to the effects of catecholamines in response to acute psychosocial stress in young men (Strahler, Rohleder, & Wolf, 2015). However, more research is needed to examine these effects in women and older participants since the results of this study are restricted to young males.

STRESS RESPONSE

Two major systems regulating the biological stress response are the hypothalamic-pituitary adrenal (HPA) axis and the sympathetic adrenal medulla (SAM) system, both of which

interact with the periphery to communicate states of danger. When a stressor is introduced into the system, the hypothalamus is activated and both axes are stimulated. As part of the SAM response, catecholamines such as epinephrine and norepinephrine are released into the blood from the adrenal medulla, and the catecholamines maintain homeostasis by regulating metabolism, heart rate, blood vessel tone, and thermogenesis at rest. In the presence of a stressor, the stress response prepares the cardiovascular and neuroendocrine systems for a fight or flight response by enhancing immune functioning in organs to fight off infections. These catecholamines initiate the fight or flight response by binding to a-adrenergic and b-adrenergic receptors, which are present all throughout the body. Therefore, catecholamines impact the functions of many systems such as the heart and lungs, inhibit the functions of the gastrointestinal tract, and increase the acuity of vision and hearing (Tsigos & Chrousos, 2002).

When a stressor is introduced, the HPA axis responds on a slower time course compared to the SAM response. The stress response of the HPA axis is initiated in the paraventricular nucleus (PVN) of the hypothalamus, where axons project to blood vessels to release corticotrophin-releasing hormone (CRH). CRH binds to a blood vessel in the pituitary, which stimulates the release of adrenocorticotropic hormone (ACTH). ACTH is released from the pituitary into the blood circulation, through which it reaches the entire body, but specifically binds to the adrenal gland to release cortisol (Sorrells, Caso, Munhoz, & Sapolsky, 2009). The HPA axis acts in a negative feedback loop upon the release of cortisol, where cortisol binds to receptors in the hippocampus to terminate the HPA axis from releasing more cortisol (Jacobson, 2005).

SNS AND AGE

Compared to other stress hormones such as cortisol, much less is known about the aging effects of the SAM response (Heffner, 2011). There are mixed findings regarding SAM

changes with age, where high basal circulating levels of norepinephrine have been reported in older men compared to younger men (Esler et al., 1995; Kudielka, Schmidt-Reinwald, Hellhammer, Schurmeyer, & Kirschbaum, 2000), but basal levels of epinephrine have been found to be inversely related to age (Franco-Morselli, Elghozi, Joly, Di Giuilio, & Meyer, 1977; Weidmann et al., 1978). In addition to this conflicting literature, catecholamine output responses to stress have been found to decline with age (Esler et al., 2002; Seals & Esler, 2000), whereas another study did not find any age effects of catecholamines in response to stress (Aslan, Nelson, Carruthers, & Lader, 1981). Although little is known about the effects of age on sympathoadrenal activity during stress, these conflicting findings suggest that there may be external factors such as altered catecholamine sensitivity that are influencing these age-related differences in catecholamine responses to stress.

SNS AND INFLAMMATION

Specifically, altered catecholamine sensitivity may impact stress-induced inflammation. Immune cells such as lymphocytes and macrophages have surface adrenergic receptors, which bind to the catecholamines. Adrenergic receptor binding activates the transcription factor NF-κB DNA-binding activity, thereby stimulating the inflammatory response (Bierhaus et al., 2003; Wolf, Rohleder, Bierhaus, Nawroth, & Kirschbaum, 2009). The infusion of a beta-adrenergic agonist, isoproterenol, as well as exposure to an acute psychosocial stressor, induces short-term increases in cytokines such as IL-6, thus having pro-inflammatory effects (Mohamed-Ali et al., 2001; Steptoe et al., 2007). Cytokines, which are molecules secreted by the white blood cells, are released by immune system to amplify local immune responses to eradicate pathogens, and eventually trigger neuroendocrine responses that return the system to a resting state. While the catecholamines secreted from the SAM axis have pro-inflammatory effects mediated by NF- κ , but they also display anti-inflammatory effects. Catecholamines have been found to suppress mitogen-stimulated production of pro-inflammatory cytokines in the cell

culture. This mechanism could be explained as protecting the host from the detrimental effects of pro-inflammatory cytokines when the inflammatory response is already activated (Elenkov, Wilder, Chrousos, & Vizi, 2000). These divergent roles of catecholamines involving pro-inflammatory and anti-inflammatory effects can potentially be explained by adrenergic signaling suppressing or activating the inflammatory cascade depending on by the time of incubation or the state of the cell (Sapolsky, Romero, & Munck, 2000) .

GCS INFLUENCE ON INFLAMMATION

In addition to catecholamines, GCs also regulate stress-induced inflammation, exerting primarily anti-inflammatory effects. HPA-axis activation results in release of the primary glucocorticoid (GC), cortisol, into the blood. Upon reaching their target cells, GCs bind to receptors, which translocate to the cell nucleus and bind to glucocorticoid response elements (GREs), where they regulate gene transcription. GC binding negatively regulates the expression pro-inflammatory cytokines such as IL-1b, IL-6, and TNF-α, which results in a down-regulation of inflammatory response. Anti-inflammatory properties of GCs include suppress maturation, differentiation and proliferation of immune cells (Sapolsky et al., 2000). The anti-inflammatory effects of GCs were originally thought to prevent the inflammatory response from being overactive and causing damage to the host, but deviations from the typical GC secretion patterns also result in negative health outcomes (Sapolsky et al., 2000). Increased cortisol levels are known to result from chronic psychological stress, which overly suppresses the immune system from their anti-inflammatory properties. However, prolonged exposure to chronic stress may create a predisposition towards excess inflammation since the CNS and GCs no longer properly suppress the inflammatory response. For example, long-term stressful lifestyles such as caregiving have been shown to induce a relative resistance to the anti-inflammatory properties of GCs, which eventually leads to low-grade chronic inflammation (G. E.

Miller, Cohen, & Ritchey, 2002).

Since the SNS can activate inflammatory responses in the absence of pathogens, it could be hypothesized that increases in inflammatory changes due to chronic stress occur from the changes to the stress system resulting from improper stress hormone responses. However, this does not fully explain disease susceptibility. Chronic stress has been associated with increased concentrations of IL-6, which has been linked to disruptions in psychological and physical health (Neurath & Finotto, 2011). High levels of plasma IL-6 have also been seen in individuals with psychological disruptions such as those who are depressed, cynical, distrustful, or report chronic stress, further implicating the role of psychological factors in regulating IL-6 concentrations in a more chronic state (Ranjit et al., 2007).

STRESS-INDUCED CHANGES IN GC SENSITIVITY

The increase in inflammatory markers from chronic stress should theoretically be explained by changes in stress system activity, but studies have demonstrated there is significant inter and intra-individual variability of inflammatory target tissues receiving stress signals. For example, a longitudinal study of cancer caregivers found increases in C-reactive protein (CRP), a protein that increases in response to inflammation, but no changes in basal HPA axis activity (Rohleder, Marin, Ma, & Miller, 2009). These results demonstrate that stress-induced changes in inflammation can potentially be explained by differences in the ability of target tissues to receive a given stress hormone signal. Alterations in GC sensitivity have in fact been found in response to acute psychosocial stress, exercise, chronic stress, and posttraumatic stress disorder (Rohleder, Wolf, & Wolf, 2010). Sex differences in GC sensitivity of pro-inflammatory cytokine production after a psychosocial stressor have been implicated, where females showed decreases in GC sensitivity and increased LPS-induced cytokine production post-stress (Rohleder, Schommer, Hellhammer, Engel, & Kirschbaum, 2001).

Understanding the effects of GC sensitivity on inflammation helps to shed light on the connections between psychosocial stress and potential health outcomes, but only addresses one aspect of the stress response system.

CATECHOLAMINE SENSITIVITY

While studying inflammatory responses to catecholamines is important for providing a more complete understanding of the stress hormones' involvement in inflammation, only a few studies have examined these responses in healthy subjects. A recent study set out to examine the effects of repeated acute stress exposure on catecholamine sensitivity of inflammatory cytokine production (Strahler et al., 2015). Reduced sensitivity of TNF-α, but not IL-6, was found for the inhibitory signals of epinephrine and norepinephrine, but no evidence of habituation of these effects was found. While this study provides an expansion in knowledge from GC-dependent effects to catecholamine-dependent effects, the divergent findings may be limited by a small sample size (N=20) and the fact that the data was obtained from only young men. In order to apply TNF-α catecholamine sensitivity changes to the general population, a larger sample is needed since conclusions from this study cannot be applied to women as well as older participants.

Although it is known that GC sensitivity changes in response to stress, measuring GC sensitivity only addresses one of the major stress systems involved with regulating inflammation. Thus, it is important to investigate whether catecholamine sensitivity also changes in response to stress, since catecholamines are also important regulators of immune response to stress. By identifying how catecholamines regulate the inflammatory response, the ways in which stress results in severe health consequences will be better understood. Gender differences among inhibited TNF-α production from norepinephrine to repeated stress will also be assessed since there are strong gender dimorphisms among immune disorders. Men are

more susceptible to bacterial and viral infections (Offner, Moore, & Biffl, 1999), where women are more affected by autoimmune diseases, with female to male ratios of 4:1 for rheumatoid arthritis(Da Silva & Hall, 1992), 9:1 for systemic lupus erythematous (Lahita, 1997) and 19:1 for autoimmune thyroid disease (Wilder, 1998).

To this end, this study examines how catecholamine sensitivity in response to an acute psychosocial stressor differs by sex and age. Therefore, *in vitro* sensitivity of inflammatory cytokine production to the effects of norepinephrine will be examined. Since habituation to repeated stress has been suggested to be an adaptive mechanism (McEwen, 1998) and has been shown for GC stress responses but not catecholamine stress responses (Kirschbaum et al., 1995), the stability of catecholamine sensitivity responses to repeated psychosocial stress will also be examined. Thus, it is hypothesized that 1) the TSST will be effective at inducing a SNS-mediated stress response on both days, 2) Amylase responses and TNF-α production will not habituate to repeated stress 3) there will be changes in TNF-α production from the suppressive effects of norepinephrine 4) Men and women will differ in inhibited TNF-α production from norepinephrine between days, 5) as well as older and younger groups.

METHODS

PARTICIPANTS

Whole blood samples were obtained from both female and male participants (N=35, 65.7% female). Participants were divided between younger and older participants, where 17 of the participants were younger, and 18 older (Table 1). Gender distributions among age groups can be seen in Table 1. Participants were recruited through advertisements and local newspapers, and eligibility, demographics and health behaviors were assessed through telephone screenings. Individuals were excluded if they reported acute or chronic medical conditions, body mass index (BMI) below 18 or above 35 kg/M^2, as well as corticosteroid or adrenergic medications, and chronic tobacco and alcohol consumption. Females were in the luteal phase of their menstrual cycle, and had normal menstrual cycles.

EXPERIMENTAL PROTOCOL

Eligible participants were scheduled for two laboratory visits on two consecutive weekdays in the afternoons, and experimental protocol was similar on both days. Participants were instructed to refrain from physical exercise, heavy lunches and alcoholic beverages on both test days. Upon arrival at the laboratory, a catheter was inserted into the non-dominant arm followed by a 45 minute resting period. Baseline blood and saliva samples were obtained after the resting period, and participants were immediately subjected to the Trier Social Stress Test (TSST) on both consecutive days. The TSST consists of a preparation period (3 min), a free speech (5 min), and mental arithmetic task (5 min) performed in front of a two-person panel and a video camera. This task is used to evoke a strong physiological stress response. Blood

samples were taken again 1, 30, 60 and 120 minutes after the TSST, thus resulting in a total of 5 blood sample collections. Saliva samples were obtained again 15, 25, 35 and 60 minutes after the TSST to track neuroendocrine response trajectories, thus resulting in a total of 5 saliva samples.

PSYCHOMETRIC ASSESSMENT

Prior to the TSST, participants completed the 10-item Perceived Stress Scale (PSS) to assess self-reported perceived chronic stress (Cohen, Kamarck, & Mermelstein, 1983). Responses are given on a Likert rating scale, scores ranging from 'never' (0) to 'very often' (4). An example of an item is: “in the last month, how often have you felt that you were unable to control the important things in your life?” Perceived stress scores were calculated by summing the scores of the items, with higher numbers representing more perceived stress and scores ranging from 0 to 40.

The Center of Epidemiological Studies Depression Scale (CES-D; Radloff, 1977) was used to assess depression symptoms. The CES-D is a 20-item questionnaire that asks participants to rate depressive symptoms over the past week on a 4-point scale ranging from 0 (rarely) to 3 (most of the time). An example item is: “I felt lonely”. Higher depressive symptoms are represented by higher scores, where a score of above 16 represents clinically significant depression.

SALIVARY ALPHA-AMYLASE ASSESSMENT

For assessment of SNS activity, salivary alpha amylase was measured using salivette collection devices (Sarstedt, Numbrecht, Germany) at the time points indicated above and was

assessed using a colorimetric assay. Amylase has been suggested as reliable indicator of stress-induced SNS responses, and provides an easy and non-invasive alternative to plasma catecholamine assessments (Rohleder & Nater, 2009).

CATECHOLAMINE SENSITIVITY ASSESSMENT

Catecholamine sensitivity was assessed with samples taken immediately before, as well as 1, 30, 60, 120 minutes after stress. *In vitro* whole blood incubation assay was set up using norepinephrine as an inhibitory agent. Therefore, catecholamine sensitivity in the current study refers to the sensitivity of stimulated production of TNF-α in whole blood.

Blood was drawn into heparin-coated vacutainers and was diluted 10:1 with saline and divided into twelve aliquots of 400 ml in cell culture pates. Whole blood was incubated with lipopolysaccharide to stimulate pro-inflammatory cytokine production. Each aliquot was co-incubated with a different concentration of norepinephrine (Kavelaars, Kuis, Knook, Sinnema, & Heijnen, 2000); (Kavelaars, van de Pol, Zijlstra, & Heijnen, 1997); (Rohleder et al., 2001). Final concentrations in the respective wells were 30 ng/mL LPS, and 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M norepinephrine, respectively. After 18 h of incubation at 37°C and 5% CO_2, plates were centrifuged for 10 min at 2000x*g a*nd 4°C and plasma supernatant was collected and stored at –80°C until analysis. IL-6 and TNF-α concentrations were measured using commercially available ELISA kits (BD Pharmingen, San Diego, CA, USA). Inter- and intra-assay CVs of TNF-α assays were below 10%, respectively.

STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS 21 for Mac OS X software packages (IBM, Chicago, IL, USA). All data was tested for normality of distribution prior to the analysis. Salivary alpha-amylase values were log-transformed since they showed non-normal

distributions. To describe the dose-response curve of norepinephrine suppression of TNF-α production, GraphPad PRISM (GraphPad Prism version 6.0b for Mac OXS, GraphPad Software, San Diego, California USA) was used to fit a linear regression for each individual dose-response curve. The slope of the regression was exported, where lower numbers, equaling steeper slopes, represent higher catecholamine sensitivity and higher numbers represent lower catecholamine sensitivity.

To test the hypothesis that the stress paradigm activates the SNS to assess the reliability of the stress responses, a 2 X 5 repeated-measures ANOVA (analysis of variance) with the within-subject factors “day” (first TSST vs. second TSST), and “sample” (saliva samples 1-5) with amylase as the dependent variable.

To test for stress-induced changes in catecholamine sensitivity, a set of a 2 X 5 X 6 repeated measures ANOVAs with the within-subjects variables “day” (first TSST vs second TSST), “sample” (blood samples 1, 2, 3, 4 and 5), and “norepinephrine” (six NE concentrations), and TNF-α as dependent variables. In addition, a 2 X 5 repeated measures ANOVA was conducted to test how well LPS stimulated cytokine production with the within-subject factors “day” (first TSST vs. second TSST), and “sample” (LPS stimulated TNF-α values from blood samples 1, 2, 3, 4, and 5). To assess the stress-related differences in the linear slope and inhibition curves of catecholamine suppression of TNF-α production, another set of two 2 X 5 repeated measures ANOVAs were computed with the within-subject factors “day” (first TSST vs. second TSST) and “sample” (linear slopes and log transformed IC50s computed across the six NE inhibited samples 1, 2, 3, 4 and 5) and TNF-α as dependent variables.

To test for the hypothesis of gender differences in norepinephrine-mediated TNF-α suppression, a 2 X 5 X 6 X 2 repeated measures ANOVA was conducted with the within-subjects variable “day” (first TSST vs. second TSST), “sample” (blood samples 1, 2, 3, 4 and 5) “norepinephrine” (six norepinephrine concentrations), “gender” (male, female), and TNF-α as

the dependent variable. Two additional 2 X 5 X 2 repeated measures ANOVAs were conducted, with the within-subjects variable “day” (first TSST vs second TSST), “sample” (linear slopes and log transformed IC50s computed across the six norepinephrine inhibited samples 1, 2, 3, 4 and 5), and TNF-α as dependent variables. These exact steps were repeated to test the hypothesis of age (older, younger) differences in TNF-α suppression to the effects of catecholamines.

RESULTS

PRELIMINARY ANALYSES

All 35 participants were healthy non-smoking men and women between the ages of 18 and 64 (Table 1). Perceived chronic stress levels were similar to average scores for this age group in the US (Cohen et al., 1983) and depressive symptoms were well below the clinical cut-offs. Males and females did not statistically differ on total PSS scores ($F_{1, 34}$= 0.74*, p*= .788), CESD ($F_{1, 34}$ = 0.42, *p*= .839), BMI ($F_{1, 34}$= 1.70, *p*= 0.201), and age ($F_{1, 34}$= 2.08*, p*= .158).

ENDOCRINE STRESS RESPONSES

Repeated-measures ANOVA of salivary alpha-amylase concentrations revealed significant increases in amylase on both testing days (time effect: $F_{5, 135}$= 11.9*, p*< .001), confirming that the acute psychosocial stress paradigm affected the SNS response (Figure 1). There were no significant differences in amylase increases by gender or age (time*gender: $F_{5, 135}$= 0.34*, p*= .859), (time*age; $F_{5, 135}$= 0.735*, p*= .599). Amylase responses did not differ significantly across days (day*time effect: $F_{5, 135}$= 0.221*, p*= .953), or by gender and age (day*time*gender*age: $F_{5, 135}$= 1.348*, p*= .248), indicating a lack of habituation of the SNS stress responses.

STRESS EFFECTS ON CATECHOLAMINE SENSITIVITY OF LPS- STIMULATED TNF- α PRODUCTION

To address the main research questions, we ran a series of repeated-measures ANOVAs testing stress and repeated-exposure-dependent alterations in *in vitro* catecholamine

sensitivity of TNF-α production. Norepinephrine was successful in significantly reducing LPS-stimulated TNF-α production ($F_{5, 175}$= 106.752, p< .001). Stress exposure resulted in significant changes in TNF-α production (time effect: $F_{20, 680}$= 2.354, p= .001), where concentrations were lower post-TSST followed by increases thereafter (Figure 2). Concentrations of TNF-α production did not habituate on the second day (day effect: $F_{5, 170}$= 0.117, p= .988). Catecholamine inhibition curves also showed a shift towards being overall lower post-TSST compared to pre-TSST (time effect: $F_{4, 144}$= 23.802, p= .006).

Males and females differed in inhibited TNF-α by norepinephrine over time between day one and day two when looking at the raw data (day*time*gender: $F_{20, 660}$= 3.697, p< .001; Figure 3) and the slope (day*time*gender: $F_{4, 128}$= 5.023, p< .001) after adding age as a covariate, but there were no gender differences in the catecholamine inhibition curves (day*time*gender: $F_{4, 140}$= 1.360, p= .251). To understand how the catecholamine sensitivity of men and women differed between days, the slopes of TNF-α inhibition between genders were examined for TSST1 and TSST2 separately, using age as a covariate. Men and women significantly differed in sensitivity on day 1 (time*gender effect: $F_{4, 148}$= 3.464, p< .01), where females showed a decrease in sensitivity and males show increased sensitivity (Figure 2). However, gender differences were not found on day 2 (time*gender effect: $F_{4, 176}$= 1.636, p< .167), where men and women both showed decreases in catecholamine sensitivity.

Older (50-64 years) and younger (18-35 years) groups did not significantly differ in catecholamine sensitivity overall ($F_{5,165}$= 0.670, p= .647), between days (day effect: $F_{5, 165}$= 0.248, p= .940), over time for each day (time effect: $F_{20, 220}$= 0.457, p= .980), or over time and between day one and day two (day*time*age effect: $F_{20, 680}$= 0.522, p= .958). The inhibition curves of TNF-α also demonstrated a lack of day by time effect across age groups (day*time*age: $F_{4, 132}$= 2.024, p= .095), as well as no effects of age among men and women

overtime on both days (day*time*gender*age: $F_{4, 132}$= 0.739, *p*= .567).

DISCUSSION

The current study set out to assess whether similar to GC sensitivity, catecholamine effects on LPS-stimulated inflammatory cytokine production is sensitive to acute psychosocial stress. In the following, each of the above findings will be discussed in detail.

As expected, the stress paradigm resulted in significant salivary alpha-amylase increases, indicating SNS reactivity. No SNS habituation effects were found since amylase levels did not statistically differ on both days, thus supporting our second hypothesis. Our hypothesis about changes in TNF-α production to the suppressive effects of norepinephrine were confirmed, where concentrations of TNF-α were higher pre-TSST and lower post-TSST, followed by increases after. As expected, gender differences in TNF-α production between days were found, where males showed increased sensitivity and females showed decreased sensitivity on day 1. However, males and females showed similar patterns of sensitivity on the second day, where they both had decreased sensitivity. Unexpectedly, older and younger adults did not demonstrate differences in TNF-α production to the effects of norepinephrine.

The lack of habituation effects with amylase is in line with previous studies using amylase as a biomarker for the SNS (Boesch et al., 2014). The changes in TNF-α production after *in vitro* stimulation with LPS and co-incubation with increasing concentrations of norepinephrine correspond with the previous pilot study that also examined catecholamine sensitivity (Strahler et al., 2015), where decreased catecholamine sensitivity for TNF-α was found. To summarize the gender differences of the present study, TNF-α was no longer affected by the suppressive effects of norepinephrine post-stressor for females on the first day, which continued to be present on the second day. For males, concentrations of TNF-a were lower

after co-incubation of increasing concentrations of norepinephrine on the first day, but these effects were reversed on the second day. Therefore, both females and males demonstrated decreased catecholamine sensitivity to repeated stress. Because the pilot study was limited to only males, the finding of gender differences in TNF-α production between days is novel. Interestingly, our results are in contrast with a recent meta-analysis that found stress-induced changes in LPS-stimulated IL-6 and TNF-α production (Steptoe et al., 2007). However, these results may be due to variations in sex hormones, where IL-6 was decreased in women in their luteal phase of the menstrual cycle post-stress, and women using oral contraceptives had increased IL-6 production (Rohleder, Wolf, & Kirschbaum, 2003). The finding that females demonstrated lower catecholamine sensitivity on both days is consistent with previous data reporting decreases in GC sensitivity and increased LPS-induced cytokine production post-stress in women (Rohleder et al., 2001), but, to the best of our knowledge, our study is the first to report gender differences among catecholamine sensitivity.

The lack of age differences in catecholamine responses and TNF-α production is not surprising due to the conflicting literature on catecholamine responses with age. Decreased catecholamine responses have been implicated with age (Esler et al., 2002; Seals & Esler, 2000), where other studies have not found age effects on catecholamine responses to stress (Aslan et al., 1981). Thus, the lack of catecholamine sensitivity effects between younger and older adults unfortunately does not help clarify age-related SNS changes to the immune system. However, since older adults have been reported to have higher basal levels of circulating norepinephrine (Esler et al., 1995; Kudielka et al., 2000), it is possible that stress responses from the TSST may have been undetected due to already high levels of norepinephrine.

The present study is the first to observe acute and short term decreases in

catecholamine sensitivity in response to repeated psychosocial stress in both females and males. The observed short-term changes should be evaluated to address the potential long-term consequences. In a healthy individual, it is thought that the endocrine system allows for the organism to deal with acute stress and its impact on various target tissues (Sapolsky et al., 2000). As mentioned above, reduced stimulatory effects of TNF-α production following stress were observed. Reduced catecholamine sensitivity, or decreased inhibitory actions of catecholamines on stimulated cytokine production may benefit the organism in mounting an effective immune response. Thus, higher levels of cytokines, despite the suppressive effects of norepinephrine may be interpreted as a beneficial counter-regulatory mechanism. However, it is speculated that frequent activation of the endocrine and immune system may lead to the wear and tear (allostatic load) of these processes (McEwen, 1998). An important consequence of allostatic load is the lack of recovery in the minutes following the stressor, which would be seen as the prolonged state of catecholamine resistance. Unrestrained inflammatory responses may lead to tissue damage or inflammatory reactions such as fever, fatigue and disease (Cizza & Sternberg, 1994; Watkins, Maier, & Goehler, 1995; del Rey, Monge-Arditi, & Besedovsky, 1998). Many studies have addressed changes in basal catecholamine sensitivity, leading to less suppression of TNF-α production among various autoimmune diseases, such as systemic lupus erythematous (Pawlak et al., 1999), and chronic fatigue syndrome (Kavelaars et al., 2000). Interestingly, no effect of TNF-α production was found in women with colitis (Langhorst et al., 2007) or inflammatory bowel disease (Lucas et al., 2007), despite autoimmune diseases being more common among women. Desensitization of beta-2 adrenergic receptors has also been found among chronically stressed Alzheimer caregivers (Mausbach et al., 2008). Thus, all of the above studies on caregivers and patients suffering from autoimmune diseases suggest a stress-related development of catecholamine resistance.

Our results showed that men had higher sensitivity on the first day, which may be interpreted as the organism protecting the body from the adverse effects of systemic elevations of pro-inflammatory cytokines. However, women are rendered more susceptible to autoimmune diseases since they illustrated decreased sensitivity on both days. These results support the gender dimorphism among immune disorders, where men are susceptible to bacterial and viral infections (Offner et al., 1999), and autoimmune diseases are more common among women (L. Miller & Hunt, 1996). However, while our results appear to support previous data on why autoimmune diseases are more prevalent among women, most data supports estradiol having an inhibitory influence on pro-inflammatory cytokines (Kramer, Kramer, & Guan, 2004; Angstwurm, Gartner, & Ziegler-Heitbrock, 1997). One study contradicts this data, where increases in TNF-α, IL-1 beta and IL-12-producing monocytes during the luteal phase among women with regular menstrual cycles were found (Bouman, Moes, Heineman, de Leij, & Faas, 2001). However, it is unclear whether the increased sensitivity of monocytes for pro-inflammatory stimuli during the luteal phase is due to increases of beta-estradiol and progesterone. Thus, future studies should focus on the underlying changes in immune cell redistribution and receptor responsivity, and how they are impacted by sex hormones. However, based on the explanations by (Heijnen, 2007), it can be theorized that the expression of alpha-adrenergic receptors are altered after repeated stress, as it is with patients suffering from autoimmune/inflammatory diseases. The changes in these receptors may be mediated by G protein-coupled receptor kinase (GRK)-2, which lead to altered norepinephrine responses and acute stress-induced changes as seen in the present study.

The results of the present study are not without limitations. First, we were not able to test whether any changes found in inflammatory regulation could be explained by the interaction of the HPA axis and SNS between two testing days. Also, the study could have benefitted from a

larger sample size, and the use of more inflammatory biomarkers as well as biomarkers of the HPA axis to gather a more well rounded understanding of stress-mediated changes to the inflammatory response. Lastly, the current study did not assess BMI effects. With a larger sample size, future studies should show whether our findings generalize to participants outside of a healthy BMI range.

Taken together, the current study revealed that acute psychosocial stress not only activates neuroendocrine systems, but also induces a significant decrease in catecholamine sensitivity of LPS-stimulated inflammatory cytokine production in whole blood. The psychosocial stress task was effective at inducing a SNS response as indicated by salivary alpha-amylase and stress-related changes in catecholamine effects on TNF-α, but no effects of habituation were found when assessed 24 hours later. Gender differences were found among TNF-α production to the suppressive effects of norepinephrine after repeated stress, where females illustrated lower catecholamine sensitivity on both days, and males showing low sensitivity only on the second day. While the supporting data on sex hormones' influence on inflammation is not entirely clear, our results provide a glimpse into how catecholamine sensitivity contributes to the gender dimorphisms among inflammatory diseases. To the best of our knowledge, our data is the first to provide gender differences with stimulated TNF-α production to the inhibitory effects of norepinephrine after repeated stress. No age differences in catecholamine sensitivity were found, which may be due to the small sample size. Future studies on stress effects on inflammatory regulation will have to include assessments of both glucocorticoid and catecholamine sensitivity in addition to measurements of circulating hormone levels. Studies focusing on the mechanisms underlying receptor responsivity and immune cell distribution will contribute to a better understanding of the role of the SNS signaling in stress-related health outcomes.

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APPENDIX A

(Figures)

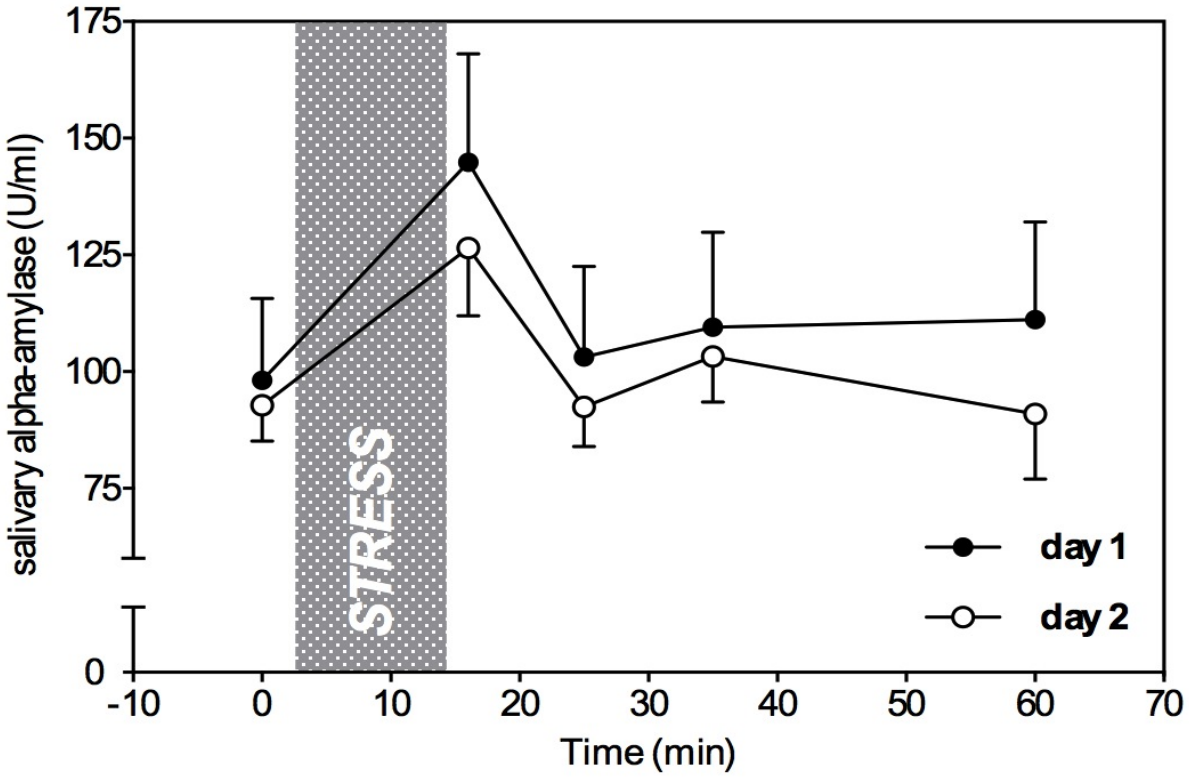

Figure 1. Salivary alpha-amylase concentrations on testing day 1 and 2 (Means ± SEM). Stress period represents TSST.

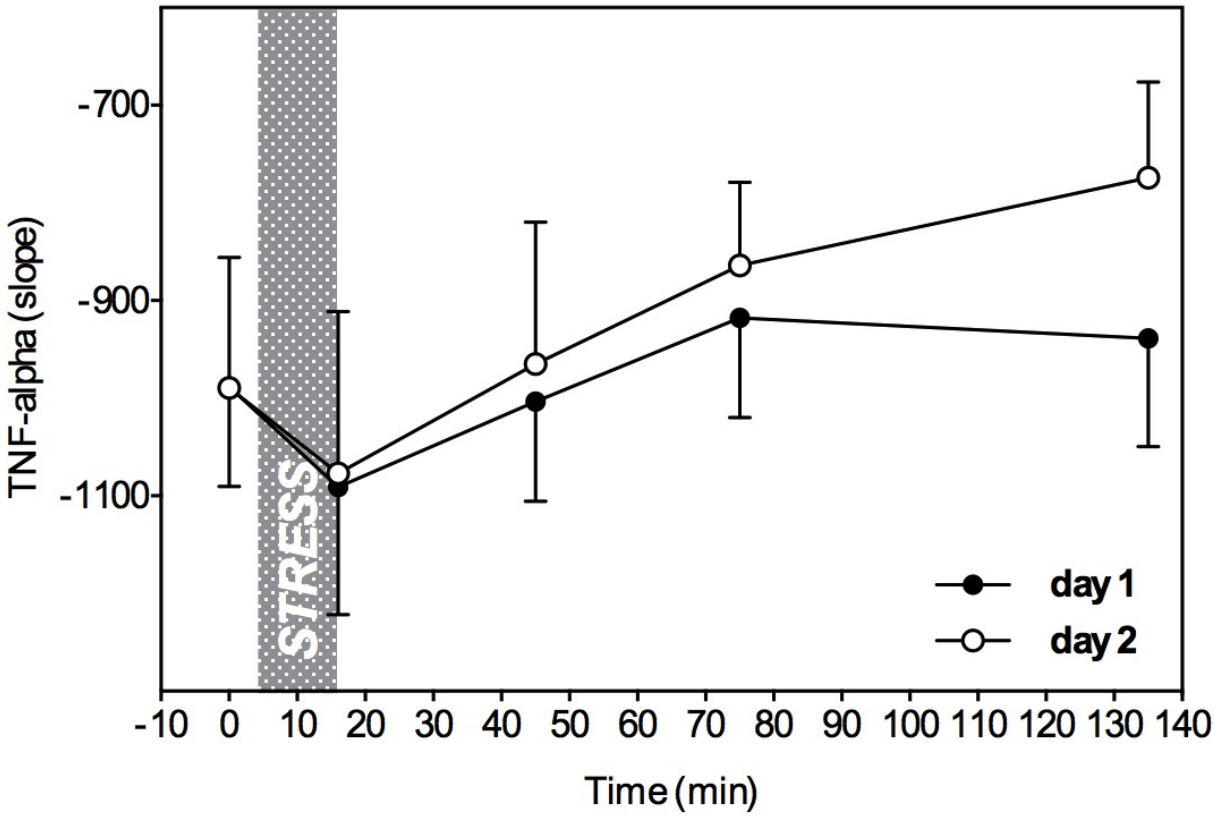

Figure 2. Average (± SEM) slopes of TNF-α in blood after in vivo stimulation with LPS and co-incubation of LPS and varying concentrations of norepinephrine.