Stress as a Link between Inflammation and Disease: Cholinergic Regulation of Inflammatory Cytokine Production and Sensitivity to Acute Stress

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ABSTRACT

Stress as a Link between Inflammation and Disease: Cholinergic Regulation of Inflammatory Cytokine Production and Sensitivity to Acute Stress

A thesis presented to the Neuroscience Department

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The stress response and relevant stress systems serve as a potential mechanism for linking inflammation with disease outcomes. Regulation of inflammation by the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) has been studied, but little is known about how the parasympathetic nervous system (PNS) mediates immunity. This research aimed to confirm the PNS as anti-inflammatory by measuring cytokine (TNF, IL-6, IL-1β, and IL-10) levels in LPS-stimulated blood incubated with increasing concentrations of acetylcholine (ACh), the primary PNS neurotransmitter. It also sought to determine whether acute stress affected ACh sensitivity of cytokine production. In both stressed (n = 4) and unstressed (n = 5) young adults, ACh was found to dose-dependently inhibit production of pro-inflammatory cytokines TNF and IL-6, but not IL-1β nor the anti-inflammatory IL-10. Acute stress,

evoked by the established Trier Social Stress Test, did not significantly modulate ACh sensitivity of cytokine production. However, data suggested an effect of stress to increase ACh inhibition of TNF and IL-6, and the reverse effect for IL-10 and IL-1β. Lack of significance was attributed to small sample size and inter-individual variability in stress response patterns. The cholinergic anti-inflammatory pathway identified here will prove a crucial target for therapy in the future. Tests of acute stress effects on ACh sensitivity should be replicated in a larger population and compared to stress effects on glucocorticoid (GC) and catecholamine (CAT) sensitivity.

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Chapter 1. Introduction

1. Specific Aims

1.1 Goals

This research aimed to better understand the relationship between inflammation and disease risk. When not properly regulated, inflammation can be disinhibited and lead to a host of adverse health consequences and age-related diseases, such as major depressive disorder and cardiovascular disease. A more comprehensive understanding of the regulatory mechanisms underlying inflammation is necessary for strategic development of therapies and precautionary measures to reduce disease risk. A strong candidate mechanism for regulation of inflammation is the stress response and the relevant stress systems. The hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) have already been identified as two crucial stress systems that regulate inflammation, but more recently a third system has been identified. The parasympathetic nervous system (PNS), usually regarded as complementary to SNS activation, may serve as a third immune feedback loop. The literature has focused on regulation of inflammation by the HPA axis and, more recently, by the SNS. However, little has been done to examine the PNS as a stress pathway that mediates inflammation. There were two specific aims of this research:

- First, it sought to confirm the PNS as anti-inflammatory. To achieve this, whole blood from healthy individuals was stimulated *in vitro* with the endotoxin

lipopolysaccharide (LPS) and incubated with increasing concentrations of acetylcholine (ACh), the primary PNS neurotransmitter. Plasma levels of inflammatory cytokines (TNF, IL-6, IL-1β, and IL-10), as a function of ACh dosage, indicated cholinergic regulation of inflammation.

- Second, this work aimed to determine whether acute stress modulated ACh inhibition of inflammation, by measuring ACh sensitivity of cytokine production before and after acute stress exposure.

1.2 Hypotheses

In accordance with an anti-inflammatory effect of the PNS (Borovikova et al., 2000), ACh levels were expected to be negatively correlated with production of pro-inflammatory cytokines, in a dose-dependent fashion. Thus, ACh was hypothesized to suppress TNF, IL-6, and IL-1β, and stimulate release of the anti-inflammatory IL-10. Although the effect of acute stress, elicited by the Trier Social Stress Test (TSST), is subject to inter-individual differences, we expected a reduced ACh sensitivity. Compared to blood drawn before stress, blood taken after the TSST was thus expected to have a greater inhibition of inflammation by ACh.

2. Inflammation and Health

2.1 Basics of Inflammation

Characterization of the inflammatory response over the past half-century has elucidated its importance as an element of the immune system. Triggered by infection or injury, inflammation is defined as the body's efforts to repair injured tissue and promote

healing of affected areas (Sell and Max, 2001). Acute inflammation is the immediate defense reaction to tissue-specific damage, and its defining clinical features are rubor (redness), calor (heat), tumor (swelling) and dolor (pain) (Meggs and Svec, 2004). These symptoms arise due to dilation of blood vessels, facilitating increased blood flow and migration of white blood cells to the damaged area, and they terminate once the cells are properly repaired (Appleton and Jacobs, 2005). Acute inflammation is short-lived, as it is mediated by several anti-inflammatory pathways that return the body to its stable state (Meggs and Svec, 2004). When inflammation is not properly attenuated, it can lead to a prolonged activation of inflammatory processes known as chronic inflammation. Importantly, chronic, low-grade inflammation can be activated in the absence of a specific pathogen or infection. As the body activates the inflammatory response without an identifiable cause, this prolonged inflammation can lead to a host of negative health outcomes (reviewed in Hotamisligil, 2006).

2.2 Inflammation and Disease

Inflammation has been linked to many disease consequences, with the implication that properly regulated inflammation is a major component of long-term health. A delicate balance of pro- and anti-inflammatory forces is requisite to ensure proper activation when needed and inactivation after the threat has ceased (Meggs and Svec, 2004). Insufficient inflammatory responses have been associated with increased susceptibility to infections and cancer, and excessive responses can lead to autoimmune disease (Pavlov et al., 2003; Sternberg, 2006; Tracey, 2002). A large body of research has linked inflammation to depression, in both animal and human studies. Exposure of

rodents to endotoxin, an inflammatory stimulus, resulted in sickness behaviors and anhedonia, a diminished experience of pleasure characteristic of depression (Yirmiya, 1996). Genetic approaches have also studied immunity in fawn-hooded rats, an accepted animal model of depression. Compared to wild-type, fawn-hooded rats showed augmented responses to the inflammatory cytokine IL-1α, indicating a compromised immune system in depressed animals (Coico and Broderick, 2005; Simmons and Broderick, 2005). One study in humans involved administration of interferon-alpha (IFN-α) to cancer patients to potentially enhance immune function (Musselman et al., 2001). Depressive symptoms, including fatigue and dysphoria, were observed in 50% of those receiving treatment, suggesting a causative role for IFN-α-stimulated inflammation in depression. The maladaptive effects of chronic low-grade inflammation are also associated with coronary heart disease (CHD) and atherosclerosis (Danesh, 1999), as well as health risks like elevated blood pressure (Bostock et al., 2011) that can lead to cardiac dysfunction and stroke. The study of inflammation is thus critical for our understanding of such life-threatening diseases.

2.3 Cytokines as Markers of Inflammation

Inflammation can be quantified by looking at changes in the secretion of pro-inflammatory cytokines, the signaling molecules of the immune system that are secreted by white blood cells (Sell and Max, 2001). These biomarkers can be measured in the bloodstream, or a participant's blood may be stimulated *in vitro* with an endotoxin, such as lipopolysaccharide (LPS), to yield an inflammatory response. Interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) are the most essential and

comprehensively studied pro-inflammatory cytokines, and C-reactive protein (CRP), secreted by the liver in response to IL-6, also serves as a common index of inflammation (Bostock et al., 2011).

2.4 Inflammation in Response to Stress

Plasma and salivary concentrations of CRP, IL-6, and TNF-α uniformly increase after exposure to acute stress (Izawa et al., 2010; Steptoe et al., 2001), and basal levels of these cytokines are elevated in people experiencing chronic stress (Kiecolt-Glaser et al., 2003; Segerstrom and Miller, 2004). To characterize the magnitude and variability of individual stress responses, researchers have looked at the relative increase in pro-inflammatory cytokine levels due to a stressor. For instance, Carpenter *et al.* (2010) found that, compared to age-matched controls, adults who experienced childhood abuse or some other early-life stress displayed a greater increase in IL-6 from baseline after acute stress exposure. Significant stress later in life may also modulate one's cytokine levels, as demonstrated in participants with fatigue after surviving breast cancer. Compared to non-fatigued survivors, those with fatigue showed a greater increase in LPS-stimulated production of IL-1β and IL-6 (Bower et al., 2007). Research in the field has also tackled the effects of subclinical depression, major depression, post-traumatic stress, aging, and obesity on the secretion of pro-inflammatory cytokines (Steptoe et al., 2007), serving as a testament to the value of biomarkers toward our understanding of such disorders. That cytokine levels, as markers of inflammation, are modulated by stress implicates the stress response as a possible mechanism for linking inflammation to disease.

3. Stress Systems

3.1 Stress and Allostatic Load

The maintenance of life and health are dependent on the body's abilities to respond to stressors, or changes in the environment, that threaten homeostasis (Widmaier et al., 2011). The coordinated processes, both physiological and psychological, that counteract these stressors are collectively known as the stress response (Schacter, 2010). Though the stress response was initially characterized in Hans Selye's "general adaption syndrome" as being activated regardless of the type of stressor encountered (Selye, 1978), there is some evidence for specificity of the response (reviewed in Tsigos and Chrousos, 2002). Recently the notion of allostasis has broadened the understanding of the dynamic equilibrium maintained by the body. Allostasis posits that bodily systems maintain stability by themselves being able to change, such as the need to sustain an elevated heart rate and increased oxygen flow in the prototypical fight-or-flight response (McEwen and Lasley, 2002) Over time, improper functioning of allostatic systems, as is the case in chronic activation of the stress response, can lead to a general wear-and-tear known as allostatic load (McEwen and Lasley, 2002). Build-up of allostatic load is associated with a variety of disorders, including hypertension and cardiovascular conditions, and serves as a theoretical framework for linking stress and disease (for discussion see McEwen, 1998).

3.2 HPA Axis

The response to stress involves activation of neuroendocrine, autonomic, metabolic, and immune system components (Sell and Max, 2001). The hypothalamic-

pituitary-adrenal (HPA) axis represents a critical neuroendocrine system that regulates the stress response. As detailed by Zillmer (2008), perception of stress causes neurons in the paraventricular nucleus of the hypothalamus to release corticotropin-releasing hormone (CRH), which triggers the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary; ACTH then stimulates the adrenal cortex to release glucocorticoids (GCs) into the bloodstream. In a negative feedback mechanism, GCs (cortisol in humans, or corticosterone in rodents) inhibit the HPA axis at the level of the hypothalamus and pituitary to suppress CRH and ACTH release, respectively. Two distinct types of intracellular GC receptors mediate this feedback: the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) (Reul and de Kloet, 1985). MRs bind tightly to endogenous GCs, produced naturally by the body, and so they quickly saturate when stress initiates release of GCs (Reul and de Kloet, 1985). Though GRs show a stronger affinity for synthetic GCs such as dexamethasone, they are the primary mediators of HPA axis down-regulation since they are available to respond to local changes in GC levels (Pariante and Miller, 2001).

3.3 Autonomic Nervous System: SNS and PNS

Also important in the response to stress are the two divisions of the autonomic nervous system, the sympathetic (SNS) and parasympathetic (PNS) nervous systems Together, the SNS and PNS regulate cardiovascular, gastrointestinal, respiratory and other systems by a rapid, involuntary mechanism (Kring, 2010). The fight-or-flight response to a stressor is associated with activation of the SNS, which accelerates heart rate, relaxes bronchi, and inhibits the digestive system (Schacter, 2010). Signaling within

the SNS is achieved through neural projections from the spinal cord to peripheral organs through pre- and post-ganglionic fibers, which release acetylcholine and norepinephrine, respectively (Widmaier et al., 2011). When activated by stress, the SNS immediately stimulates the adrenal medulla to produce epinephrine and norepinephrine (Tsigos and Chrousos, 2002). Complementary to SNS function are the actions of the PNS, which return the body to its normal resting state by slowing heart rate, contracting bronchi, and stimulating digestion (Schacter, 2010). Like the SNS, the PNS uses pre- and post-ganglionic pathways to signal to target tissues, but unlike the SNS it uses acetylcholine in both signaling directions (Widmaier et al., 2011).

3.4 Chronic Stress and Disease Correlations

Chronic psychological stress is associated with various life-threatening diseases. The prevalence of stressful events makes the issue of controlling stress even more pressing: 25-50% Americans experience some serious psychological trauma in their lives (Acierno, 1999). Though one cannot conduct true experimental studies demonstrating that stress causes disease, evidence for a mediatory role of stress in disease is staggering in correlational studies. A nationwide prospective cohort study in Denmark determined that intense psychological stress, brought on by the death of a child, significantly increased the risk of myocardial infarction (Li et al., 2002). Another study showed that, in primates, chronic social stress also has deleterious effects on the heart (Kaplan et al., 1982). Monkeys exposed to long-term social stress, due to changing community hierarchies, had increased injury to blood vessels in the heart, leading to increased clot formation and higher risk of heart attack. Chronic stress has also been associated with

ulcers, as excessive levels of cortisol result in thinning of the stomach lining and greater risk of gastric ulcer formation (Bremner, 2002). A recent review has implicated stress in the pathogenesis and progression of four major diseases: clinical depression, cardiovascular disease (CVD), human immunodeficiency virus (HIV)/AIDS, and cancer (Cohen et al., 2007). In other cases, the psychological stress brought on by an existing disorder may exacerbate symptoms. For example, diabetes-related stress results in higher blood sugar and more difficult management of Type-2 diabetes (Morris et al., 2011). Understanding how stress leads to these adverse health consequences is the major driving force behind this field of research.

3.5 Studying Acute Stress

Despite the recognition of chronic stress as a major health risk, experimental research is limited to what can be achieved in the laboratory. Since induction of chronic stress would require months and it would be difficult to establish and maintain measures of control over such a time span, researchers have focused on methods of inducing and monitoring responses to acute stress in the laboratory. Another challenge is constructing a task that reliably activates the stress systems of all people, as interindividual variance, specifically in the cortisol response, is well-documented (Berger et al., 1987; Kirschbaum et al., 1992; Oswald et al., 2006). The Trier Social Stress Test (TSST) plays on two elements known to be psychologically stressful to humans: anticipatory cognitive appraisal and social evaluation (Gaab et al., 2005). Developed in 1993 at the University of Trier, Germany, the TSST involves the participant walking into a room with 2-3 "evaluators" sitting at a desk. The participant is led to believe that these evaluators

comprise a selection committee for his ideal job, and he will have to prepare a speech to present to them. The participant is first given five minutes to plan his speech (anticipation period), then five minutes to present his speech to the evaluators, in front of a microphone and camera, and another five minutes to complete a difficult arithmetic task (testing period). The evaluators maintain a neutral disposition, speaking in monotone and only to introduce the next task, thus allowing the participant to talk interrupted for as long as possible. Though several other measures have incorporated public speaking or mental arithmetic into an acute stress paradigm, the TSST has been shown to reliably activate the HPA axis and lead to significant increases in heart rate, ACTH, serum and salivary cortisol, and other stress hormones (Kirschbaum et al., 1993). As such, the TSST serves as the best general model for studying psychosocial stress in controlled laboratory experiments today (Dickerson and Kemeny, 2004).

4. Regulation of Inflammation by Stress Systems

Each of the stress systems (HPA axis, SNS, and PNS) mediates peripheral inflammation in response to acute and chronic stress. The literature has focused on HPA axis regulation of inflammation, while relatively little has been done in reference to the SNS and PNS as two other stress systems that control inflammation.

4.1 HPA Regulation of Inflammation

As the terminal product of the HPA axis, GCs act to downregulate this neuroendocrine system by inhibiting the transcription factor NF-κB (Scheinman et al., 1995). This inhibition of NF-κB also acts to suppress the immune system by inhibiting

the synthesis of pro-inflammatory cytokines (such as TNF, IL-1, and IL-6) and activating the synthesis of anti-inflammatory cytokines (such as IL-10) (summarized in Webster et al., 2002). Thus, GCs are one of the strongest anti-inflammatory hormones in the body, facilitating the return to homeostasis after the threat has passed (Ruzek et al., 1999). Dysregulation of GC signaling has been associated with a variety of disease outcomes and can manifest itself by irregular circulating GC levels or alterations in glucocorticoid receptor (GR) activity (Raison and Miller, 2003).

Over the past fifteen years, research has shown that exposure to chronic stress changes GC sensitivity of inflammatory tissues (for detailed review, see Rohleder, 2011). In one study, individuals caring for a spouse with dementia were found to have an excess of circulating GCs and an overactive HPA axis (Vedhara et al., 1999). After receiving the influenza vaccine, these chronically-stressed individuals showed decreased antibody production, indicating an immunosuppressive effect of the HPA axis. Conversely, women exposed to early life stressors like childhood abuse exhibit hypocortisolism, involving lower basal cortisol levels and a reduced cortisol response to ACTH (Heim et al., 2001). Hypocortisolism has also been observed in individuals with chronic fatigue syndrome, a disorder characterized by a persistent or recurrent fatigue lasting at least six months (Demitrack et al., 1991). It is thus clear that chronic stress disrupts the delicate balance of circulating GC levels necessary for proper HPA axis function.

Chronic stress can lead to long-term GC resistance, impairing the ability of the HPA axis to suppress inflammation (Miller et al., 2002). A reduced responsiveness to GCs, or lowered GC sensitivity, has been reported in studies of depression both *in vivo* and *in vitro* (discussed in Raison and Miller, 2003). The lymphocytes of major

depressives also showed GC resistance, underscoring the connection between the immune system and HPA axis (Wodarz et al., 1991). Based on evidence that the pro-inflammatory cytokines impair GR function, localized GC resistance has been hypothesized to be a direct action of cytokines on GRs during the inflammatory response (Pariante et al., 1999). Buske-Kirschbaum *et al.* (2010) studied the relation of the blunted HPA axis responsiveness in atopic syndrome to the severity of acute allergic reaction. Interestingly, they found that more blunted HPA responses correlated with more severe inflammation. Another research group studied the immune systems of people caring for a family member with cancer (Rohleder et al., 2009). Over a year, the HPA axis response of these participants did not change, but GC resistance was observed and elevated CRP levels revealed a corresponding increase in systemic inflammation. Examination of relatives of brain cancer patients, also presumed to be under chronic stress, similarly showed normal HPA axis activity but increased levels of CRP and other pro-inflammatory cytokines compared to unstressed controls (Miller et al., 2008). In sum, recent findings strongly suggest that chronic stress induces GC resistance, which is relevant for HPA axis regulation of inflammation.

The acute stress effects on GC sensitivity of inflammatory markers are less clearly defined. The Sheridan research group has investigated the effects of social stress in mice using the social disruption model (SDR) (Sheridan et al., 2000). Mice that were stressed showed significant HPA activation, and measurement of GC sensitivity of spleen cells revealed a GC resistance after repeated exposure to the SDR stress (Avitsur et al., 2002; 2001). In humans, standardized stressors such as the Trier Social Stress Test (TSST) have been shown to reliably activate the HPA response (Kirschbaum et al., 1993).

Steptoe *et al.* (2007) recently conducted a meta-analysis of thirty studies measuring inflammatory cytokine levels in humans after exposure to an acute psychological stressor, and the majority of included studies used the TSST or some modification of its public speaking or arithmetic task. A consistent finding across these studies was elevated circulating levels of the pro-inflammatory cytokines IL-6 and IL-1β following acute stress. However, *in vitro* mitogen-stimulated production of cytokines, primarily by the endotoxin lipopolysaccharide (LPS), found significant individual variability of inflammatory factors due to acute stress. For instance, Rohleder *et al.* (2002) have reported an acute stress-induced increase in GC sensitivity in young but not older men. The reduced GC sensitivity in older men was partially reversed by testosterone treatment, suggesting that changes in GC sensitivity to inflammatory tissues may be dependent on sex steroids in addition to age. In all, much has yet to be determined regarding acute stress effects on GC sensitivity across multiple populations.

4.2 SNS Regulation of Inflammation

The sympathetic nervous system (SNS) represents another stress system responsible for regulation of inflammation, through the catecholamines epinephrine and norepinephrine. Unlike the HPA axis, which is characteristically anti-inflammatory, the SNS has been shown to have both pro- and anti-inflammatory effects (Widmaier et al., 2011). This dual role of the SNS in regulating inflammation allows for activation of some target tissues and inhibition of others by the pre- and post-ganglionic signaling described earlier. As such, the SNS is ideally suited for the fight-or-flight response to a threat for its activation leads to increased perfusion of the heart and release of glucose

from the liver, while simultaneously depressing digestive and other organs (Pavlov et al., 2003). A primary mechanism by which the SNS exerts pro-inflammatory effects is in the catecholamine facilitation of DNA-binding activity of NF-κB, the main inflammatory transcription factor (Bierhaus et al., 2003). Further evidence of catecholamines as pro-inflammatory came from a study by DeRijk *et al.* (1994) that found that administration of epinephrine in rats resulted in a dose-dependent increase in plasma IL-6. However, *in vitro* studies reveal the anti-inflammatory function of the SNS, as catecholamines suppress LPS-stimulated cytokine production (van der Poll et al., 1994). It has been hypothesized that the differential effects of the SNS on inflammation are dependent on the time course and current state of the cell (Rohleder and Wolf, manuscript in preparation). Specifically, adrenergic signaling initiates the inflammatory response in previously inactive immune cells, while it inhibits inflammation in cells that are already activated.

Studies of chronically stressed individuals have been used to determine the chronic stress effects of catecholamine (CAT) sensitivity of peripheral inflammation. One study investigated CAT sensitivity in a population of people with atopic dermatitis, an inflammatory skin disease known to cause significant emotional strain in families (Buske-Kirschbaum et al., 2002). Atopic dermatitis was associated with overactivation of the SNS in response to stress and a relative CAT resistance compared to normals. Another condition associated with chronic stress is chronic fatigue syndrome. Kavelaars *et al.* (2000) had shown that terbutaline, a β2-adrenergic receptor agonist, inhibited monocyte production of the pro-inflammatory TNF-α and inhibited production of the anti-inflammatory IL-10; the regulation of both of these cytokines is disrupted in patients

with chronic fatigue syndrome. The notion that chronic stress induces CAT resistance was further supported by looking at β2-adrenergic receptors in chronically stressed caregivers (Mausbach et al., 2008). Following these participants for five years, Mausbach *et al.* found that the stress of caregiving for a spouse with Alzheimer's disease was associated with decreased adrenergic receptor sensitivity, or CAT resistance. Interestingly, increased personal mastery, or facility in coping with caregiving stress, was correlated with enhanced receptor sensitivity; this suggests that effective coping serves as a protective resource by counteracting CAT resistance. Recent evidence suggests that changes in CAT sensitivity are not limited to the periphery, as individuals exposed to early stress during childhood show changes in CAT sensitivity in the brain (Blair, 2010).

Little is known about how acute stress mediates SNS regulation of inflammation, as few studies have tested changes in CAT sensitivity in response to an acute stressor (Rohleder, 2011). Recent work by Kennedy *et al.* (2005) has identified the SNS as immunosuppressive. Exposure to acute stress immediately following immunization led to activation of the SNS and depletion of norepinephrine in the spleen. These reduced levels of splenic norepinephrine suppressed antibody function, thus interfering with the normal immune response. Unpublished research by Rohleder & Wolf (manuscript in preparation) specifically addressed acute stress effects of CAT sensitivity. In their experiment, healthy adults were exposed to the TSST and blood was drawn before and after stress exposure. Whole blood was stimulated with LPS and incubated with varying concentrations of catecholamines. Both norepinephrine and epinephrine were found to strongly inhibit production of TNF-α, evidencing the anti-inflammatory function of the SNS. Comparison of cytokine production before and after acute stress revealed a reduced

inhibition of TNF-α in the latter condition, suggesting that acute stress causes a short-term reduction in CAT sensitivity. Further research should work to verify these conclusions.

4.3 PNS Regulation of Inflammation

The parasympathetic nervous system (PNS) has received less attention than the HPA axis and SNS as a relevant stress system because it is not activated, but rather downregulated, in response to stress (Rohleder, 2011). The vagus nerve is the main signaling highway for communication between the brain and the peripheral organs, through afferent and efferent fibers (reviewed in Sternberg, 2006). This neurally-coordinated system has been termed the *inflammatory reflex* (Tracey, 2002), with the afferent fibers sensing inflammation and the efferent fiber acting to inhibit the inflammatory response. This anti-inflammatory efferent arm of the vagus nerve is called the *cholinergic anti-inflammatory pathway (CAP)* because acetylcholine (ACh) is the main PNS transmitter. Specifically, the CAP is mediated by the α7 subunit of the nicotinic ACh receptor, which is expressed on macrophages (Sternberg, 2006). As an element of the PNS, ACh exerts its anti-inflammatory effects by binding to and activating these receptors, inhibiting NF-κB and production of inflammatory cytokines (Babcock and Carlin, 2000). This year, it was discovered that ACh is produced by T-cells that are regulated by the vagus nerve, implicating these T-cells as another essential component of PNS immunosuppression (Rosas-Ballina et al., 2011).

The CAP was identified as anti-inflammatory in a landmark study by Borovikova *et al.* (2000). This research group found that the inflammatory response to endotoxin

(LPS) was attenuated by cholinergic agonists. Incubation of LPS-stimulated macrophage cultures with ACh dose-dependently reduced the release of pro-inflammatory cytokines such as TNF but not the anti-inflammatory cytokine IL-10. The data suggest inhibition of TNF was post-transcriptional, as TNF mRNA was unaffected by treatment with ACh. The researchers continued their study of the vagus nerve, in the same original paper, by directly stimulating the vagus nerve in rats undergoing sepsis. This electrical stimulation resulted in inhibited TNF synthesis and suppressed development of endotoxic shock. Experiments with swine, by the same research team, have also shown a protective effect of the CAP by electrical stimulation of the vagus nerve (Czura et al., 2010). Thus, experiments by Borovikova *et al.* showed that human macrophages exhibit extreme ACh sensitivity, and the vagus nerve is critical for immunomodulation.

There is currently no data regarding ACh sensitivity of inflammation to either acute or chronic stress, as research has focused on studying ACh sensitivity in the more relevant context of inflammatory diseases and sepsis (Rohleder, 2011; Tracey, 2010). For example, a recent study assessed CAP activity in patients with rheumatoid arthritis (RA), a condition of chronic systemic inflammation (Bruchfeld et al., 2010). Compared to healthy controls, those with RA showed suppressed vagus nerve activity, raising the possibility that they cannot adequately activate the CAP system. In *ex vivo* whole blood cultures stimulated with LPS, TNF production was observed in RA samples, albeit to a lesser degree than in healthy control samples; this suggests that, despite suppressed vagus nerve activity, RA immune cells still show CAP activation. Certainly, more research will need to investigate changes in ACh sensitivity to expand knowledge on the CAP and potentially target it to fight diseases associated with inflammation.

5. Summary

Recent research, especially in the past decade or so, has done much to characterize how stress mediates inflammation. Dysregulation of inflammation is associated with a variety of life-threatening disorders, including stroke, coronary heart disease, and atherosclerosis (Bostock et al., 2011; Danesh, 1999). Understanding the factors and systems that lead to the irregular inflammatory response is thus of great importance to establish preventive measures and targeted treatments. Stress provides a good candidate mechanism for explaining the inflammation-disease link since the three stress systems, the HPA axis, SNS, and PNS, have been shown to regulate inflammation (see earlier discussions of each stress system). The literature reviewed here show that GCs, as an element of the HPA axis, are critical for suppressing the inflammatory response and sensitivity to GCs is modulated by exposure to stress. Chronic stress induces GC resistance of inflammation (Miller et al., 2008; Miller et al., 2002; Rohleder et al., 2009), while changes GC sensitivity due to acute stress have been reported as dependent on age and sex steroids (Rohleder et al., 2002). Less is known about the modulatory effects of stress on SNS control of inflammation, but CAT resistance has been observed in many conditions of chronic stress (Mausbach et al., 2008). Preliminary data on CAT sensitivity due to acute stress suggests a relative CAT resistance (Rohleder and Wolf, manuscript in preparation). Even less is known about regulation of inflammation by the PNS, as a third immune feedback loop. Though the cholinergic anti-inflammatory pathway has been explained in detail by Tracey and colleagues (Rosas-Ballina and Tracey, 2009; Tracey, 2010), studies have yet to assess ACh sensitivity to stress, in either chronic or acute stress conditions. The present research therefore sought

to examine ACh sensitivity to acute stress, by first developing an appropriate *in vitro* assay (Experiments 1 and 2), then testing it in a non-stressed (Experiment 3) and an acutely-stressed set of participants (Experiment 4).

Chapter 2. Experiments

Experiment 1. Calculation of Cytokine Dilutions

The purpose of Experiment 1 was to discover the proper dilution factors for measuring TNF, IL-10, IL-1β, and IL-6 in LPS-stimulated human plasma treated with increasing concentrations of ACh.

1.1 Methods

1.1.1 Participants

One healthy male, aged 21 years, volunteered to come into the lab to give a small amount of blood. A certified phlebotomist drew 10.0mL blood into a vacutainer coated with sodium heparin (Becton Dickinson [BD]). The volunteer received no compensation.

1.1.2 Blood Processing

Under a sterile hood, a 24-well tissue culture plate was prefilled with 50µL 100 ng/mL lipopolysaccharide (LPS), 5µL 1 mM pyridostigmine (PYR) and 50µL ACh at increasing concentrations (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M). These ACh concentrations were chosen to maximize physiological relevance, for ACh can reach the mM range in mammalian tissue (Borovikova et al., 2000). Blood was immediately diluted 10:2 in saline, then 400 µL diluted blood was pipetted into each well. The tissue culture plate was placed on a shaker (TSZ-S-02, TSZ Scientific) at low speed for 5 minutes, then incubated for 18 hours at 37^{o}C (CO_2 Incubator, Sanyo). Plates were removed from the

incubator and centrifuged at 4°C and 2000*g* for 10 minutes, and 120µL plasma from each well was transferred into a storage plate that was stored at -80°C.

1.1.3 Measurement of Cytokine Concentrations

The plasma concentrations of TNF, IL-6, IL-1β, and IL-10 were measured for each ACh concentration using a standard ELISA procedure (BD OptEIA Set). Briefly, four 96-well microplates (MaxiSorp) were coated with cytokine-specific capture antibody diluted in coating buffer (0.1 M sodium carbonate, pH 9.5) and stored overnight at 4°C. Plates were washed four times with wash buffer (PBS with 0.05% tween-20), then blocked with assay buffer (90% phosphate-buffered saline [PBS], 10% fetal bovine serum [FBS]). Plates were incubated for at least one hour at room temperature, then washed four times before 100µL standard or plasma sample was pipetted into each well. After at least two hours of incubation at room temperature, plates were washed four times and working detector was added. The working detector consisted of a 1:250 dilution of the cytokine-specific detection antibody and enzyme concentrate Streptavidin-HRP (exception of 1:500 dilution for IL-1β) into assay buffer. The plates were incubated for one hour at room temperature, then washed six times before substrate solution (TMB Substrate Reagent Set, BD) was added, producing a blue color in the wells. After 30 minutes of incubation in the dark, sulfuric acid was added as stop solution, causing the wells to turn yellow. Absorbance at 450nm was measured with a microplate reader (TECAN) and corresponding software (Magellan).

Importantly, inclusion of undiluted plasma samples in the assay would result in an optical density (OD) unreadable by absorbance. Since the optimal dilution of plasma had

not previously been determined for LPS-stimulated blood treated with ACh, the samples were diluted in assay buffer according to a broad range of dilution factors (Table 1).

	TNF	IL-6	IL-1β	IL-10
Dilutions Tested	10	100	2	2
	20	200	10	10
	30	300	100	100
	40	400	1000	1000
	60	600		
Best Dilution	50	1000	500	10

Table 1. Library of tested dilutions of plasma in assay buffer. In order for the concentration of each cytokine (TNF, IL-6, IL-1β, IL-10) to be properly read, the plasma needed to be diluted to yield ODs within the range of the standard curve for that cytokine.

1.2 Results

The optimal dilution factors were chosen based on which dilution yielded the best range of ODs, as defined by roughly the middle of the standard curve for that cytokine. Measurement of TNF yielded ODs for the standards ranging from 15 - 469 pg/mL, meaning the ideal dilution for TNF measurement should result in an OD of approximately 150 pg/mL. The 1:40 and 1:60 dilutions of plasma yielded ODs of 220 and 99 pg/mL, so the optimal dilution was set at 1:50 for TNF. The ODs for IL-6 standards ranged from 9 - 317 pg/mL, but all of the tested dilutions of ACh-treated plasma yielded ODs too high for measurement. Therefore, the optimal dilution for IL-6 was set at 1:1000 considering not even 1:600 had produced readable results. The IL-1β standards yielded ODs of 7 - 216 pg/mL, calling for an ideal OD of about 50 pg/mL. The observed ODs were 150 -170 pg/mL for 1:100 and 17 - 22 pg/mL for 1:1000, suggesting

a 1:500 dilution of plasma for future IL-1β measurement. Lastly, the ODs for IL-10 standards ranged from 15 - 437 pg/mL, meaning the ideal dilution should produce about 100 pg/mL IL-10. In fact, the 1:10 plasma dilutions yielded 79 - 89 pg/mL IL-10. In short, the best dilution factors for measurement of TNF, IL-6, IL-1β, and IL-10 were determined to be 1:50, 1:1000, 1:500, and 1:10, respectively (Table 1).

1.3 Conclusions

The goal of this experiment was to establish the appropriate dilutions of plasma that would produce readable OD measurements for each of TNF, IL-6, IL-1β, and IL-10. By testing a number of dilution factors, the ideal dilution factor was successfully identified for all four cytokines. As this experiment consisted of a single volunteer, and the ACh-treated blood was diluted according to various factors, it was not appropriate to begin looking at the effect of ACh on cytokine levels. However, now that the ELISA was prepared with the proper dilution factors, a second experiment with more volunteers could elucidate the differences in cytokine levels as a function of ACh concentration.

Experiment 2. Effect of ACh in Non-Stressed Individuals

The purpose of Experiment 2 was to obtain preliminary data for the effect of ACh, at varying concentrations, on cytokine levels in individuals not exposed to acute stress. In accordance with its anti-inflammatory effect, ACh was expected to dose-dependently inhibit the pro-inflammatory cytokines TNF, IL-6, and IL-1β, and potentially stimulate the anti-inflammatory IL-10.

2.1 Methods

2.1.1 Participants

Six healthy volunteers, divided evenly by gender, were recruited to come into the lab to have 10.0mL blood drawn by a certified phlebotomist. All volunteers were acquaintances of the author and came into the lab in the afternoon. Exclusion criteria included diagnosis of mental and other illnesses, prescribed medications, and trypanophobia, in order to ensure general health and a stress-less blood draw (see Appendix). No monetary compensation was provided, but volunteers did receive a short explanation of the present research.

2.1.2 Blood Processing and Measurement of Cytokine Concentrations

The six blood draws were spaced out by 15 minutes or longer. Blood was processed immediately and incubated with LPS, PYR, and increasing concentrations of ACh, per the protocol detailed in Experiment 1. Plasma concentrations of TNF, IL-6, IL-1β, and IL-10 were measured by ELISA, using the respective dilution factors identified in Experiment 1 (Table 2). Statistical analysis was conducted with PASW software packages (SPSS 18, IBM). For each cytokine, a repeated-measures ANOVA was run with ACh concentration as a within-subjects variable and cytokine concentration as the dependent variable, to test for the effect of ACh on cytokine concentration. Gender differences were not considered, due to the small sample size. All significance values were computed using the Greenhouse-Geisser procedure.

	IL-10	TNF	IL-1β	IL-6
Target Dilution	1:10	1:50	1:500	1:1000
Assay Buffer (µL)	270	240	225	495
Plasma (µL)	30	60 (from IL-10)	25 (from TNF)	5 (from IL-10)
Gross Volume (µL)	300	300	250	500
Remaining Volume (µL)	235	275	250	500

Table 2. Dilutions of plasma in assay buffer. Higher dilutions were achieved through a two- or three-step mechanism. At least 200µL of the final, diluted samples were required for the ELISA.

2.2 Results

This experiment aimed to determine whether ACh modulated cytokine levels in non-stressed volunteers, by incubating their LPS-stimulated blood with increasing concentrations of ACh and measuring the resulting plasma cytokine concentrations. ACh was observed to dose-dependently inhibit gross concentrations of TNF and IL-1β, though repeated measures ANOVAs did not show these results to be significant (Figure 1a, d). Inconsistent effects of ACh were observed for concentrations of IL-6 (Figure 1c). In contrast, plasma IL-10 concentrations were significantly affected by ACh ($F = 4.593$, $p < 0.05$) exhibiting slight stimulation (Figure 1b).

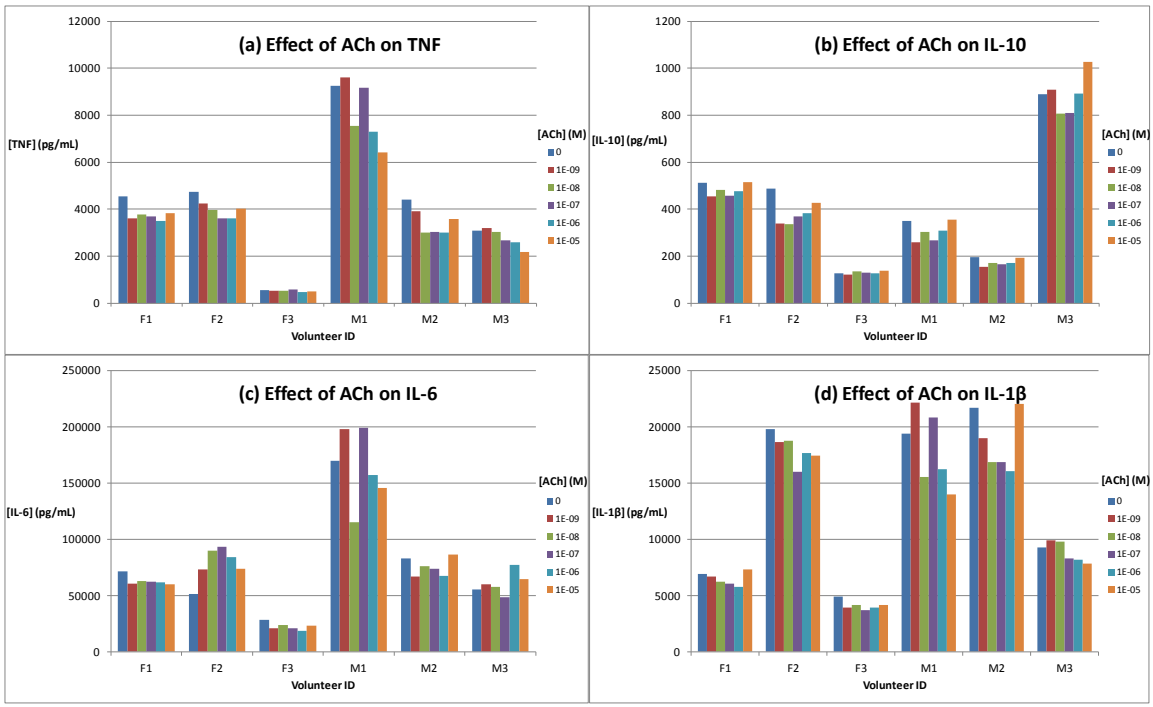

Figure 1. The effect of increasing ACh concentrations on cytokine levels in non-stressed individuals (n = 6). Plasma TNF **(a)**, IL-10 **(b)**, IL-6 **(c)**, and IL-1β **(d)** levels are shown for each volunteer (F = female; M = male). ACh appeared to inhibit the pro-inflammatory cytokines TNF and IL-1β, but a repeated measures ANOVA revealed no main effect of ACh on cytokine concentration ($F = 4.070$, $p = 0.073$; $F = 1.613$, $p = 0.264$, respectively). ACh did not affect IL-6 concentrations ($F = 0.340$, $p = 0.696$). A significant main effect of ACh on IL-10 was observed ($F = 4.593$, $p < 0.05$), suggesting a slight stimulatory effect.

2.3 Conclusions

This experiment generated promising data supporting ACh modulation of cytokines in a non-stress situation. Though not all results were significant, qualitative data suggested that TNF, IL-6, IL-1β, and IL-10 were responsive to ACh. As expected,

ACh inhibited the pro-inflammatory TNF and IL-1β, and a slight stimulation of the anti-inflammatory IL-10 was observed. These trends are in line with the research implicating the ACh pathway as anti-inflammatory. In order to amplify the effect of ACh, it was proposed to include additional, higher concentrations of ACh in future assays. As the highest concentration of ACh tested in this experiment was 10^{-5} M, these higher concentrations were suggested to be 10, 100, and 1,000 times as concentrated, corresponding to 10^{-4}, 10^{-3}, and 10^{-2} M. Furthermore, testing the concentrations of PYR (previously set at 1 mM) could illuminate any interactive effects with ACh on cytokine production, or perhaps even an ACh-independent effect of PYR. Thus, a future assay should include additional conditions with no PYR and more highly concentrated (10 mM) PYR.

Experiment 3. Effects of ACh and PYR in Non-Stressed Individuals

The purpose of Experiment 3 was to manipulate the concentrations of ACh and PYR in order to determine the effects of each chemical in a non-stressed system and ultimately determine the appropriate concentrations of each with which to proceed in future assays.

3.1 Methods

3.1.1 Participants

Five healthy volunteers, ranging in age from 19-22 years, were recruited for 10.0mL blood draws. There were two females and three males, and each was screened

according to the same exclusion criteria as Experiment 2 (see Appendix). Volunteers were not compensated, but they were informed about the goals of the present research.

3.1.2 Blood Processing and Measurement of Cytokine Concentrations

Each blood sample was incubated with 50µL LPS, 8 increasing concentrations of ACh (0, 10^{-8} - 10^{-2} M), and either of three PYR conditions. In yesPYR, 5µL 1 mM PYR was added as in Experiments 1 and 2 (see *Section 1.1.2*); in hiPYR, 5µL 10 mM PYR was added; and in noPYR, no PYR was added. The measurement of plasma cytokine concentrations was conducted as described in Experiment 1 (see *Section 1.1.3*), using appropriate dilution factors for the plasma samples (Table 1). PASW software packages were used for statistical analysis of cytokine concentrations. Repeated measures ANOVAs were run for each cytokine, with ACh concentration and PYR concentration as within-subjects factors and cytokine concentration as the dependent variable. Gender differences were ignored. All significance values were computed under Greenhouse-Geisser specifications.

3.2 Results

Plasma concentrations of TNF, IL-10, IL-6, and IL-1β were measured from LPS-stimulated blood treated with increasing concentrations of ACh and 1 mM, 10 mM, or no PYR, which had been drawn from non-stressed individuals (Figure 2). Unlike in Experiment 2, where ACh only exhibited speculative modulatory effects, here the effect of ACh was determined to be significant for all four cytokines (Table 3). In the absence of PYR (e.g. condition noPYR), ACh was observed to dose-dependently down-regulate

TNF, IL-6, and IL-10 but not IL-1β. PYR was observed to significantly affect the concentrations all cytokines but IL-6. The presence of PYR (e.g. conditions hiPYR and yesPYR) stimulated TNF and IL-1β, and suppressed IL-10. Significant ACh by PYR interactions were observed for all cytokines. However, this interaction did not yield consistent directional effects for any cytokine.

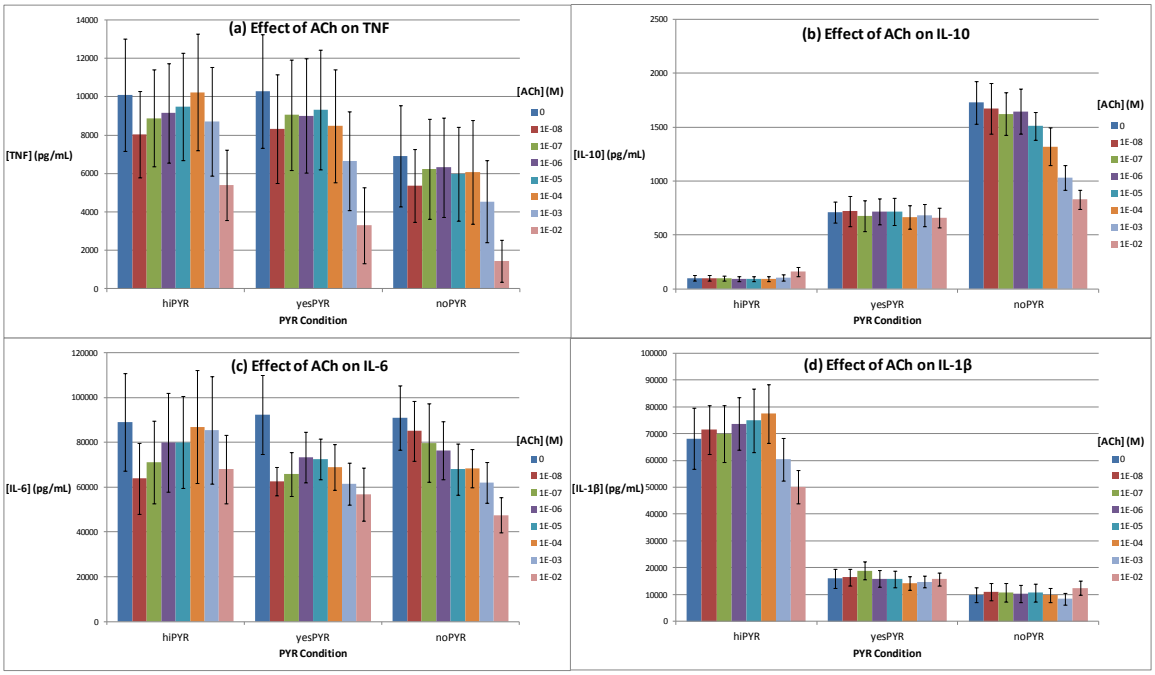

Figure 2. Cytokine levels were modulated by ACh and PYR concentration in the blood assay. Average levels, from the plasma of five human volunteers, of TNF **(a)**, IL-10 **(b)**, IL-6 **(c)**, and IL-1β **(d)** are shown for each PYR condition. An interactive effect of ACh and PYR is evident in the measurement of all four cytokines. Error bars are standard errors of the mean.

	TNF		IL-1β		IL-6		IL-10	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
ACh	15.89	0.013	6.53	0.034	8.05	0.015	13.23	0.007
PYR	21.98	0.001	60.19	0.001	1.52	0.285	75.25	< 0.001
ACh*PYR	2.482	0.122*	12.24	0.004	2.965	0.107*	20.98	0.001

Table 3. ACh and PYR modulate cytokine production independently and interactively. Repeated measures ANOVAs under Greenhouse-Geisser specifications revealed a significant main effect of ACh on all cytokines and of PYR on all but IL-6. A significant interactive effect of ACh and PYR was discovered for all cytokines (* $p < 0.05$ under Huynh-Feldt specifications).

3.3 Conclusions

The data suggest that ACh and PYR modulate cytokine concentrations both alone and interactively in a non-stressed system. The finding that ACh inhibits pro-inflammatory TNF and IL-6 production is consistent with the preliminary results from Experiment 2 and the anti-inflammatory function of ACh. Interestingly, ACh also dose-dependently inhibited the anti-inflammatory IL-10, contrary to previous findings (Borovikova et al., 2000). Even more unexpected was the main effect of PYR as a stimulator of pro-inflammatory cytokines. As an acetylcholine-esterase inhibitor, PYR was expected to effectively act as more ACh and therefore only have an interactive effect with ACh. Instead, PYR moderately increased TNF, while strongly stimulating IL-1β and inhibiting IL-10. Importantly, this independent effect of PYR indicated that future assays should avoid using high concentrations of PYR to prevent over-stimulation of cytokines, or inhibition in the case of IL-10. Taken together, this experiment established

ACh as an inhibitor, and PYR as a stimulator, of inflammation in a non-stress system. Still, the influence of ACh in response to stress remained untested in a human system.

The manipulation of ACh and PYR concentrations in this experiment also allowed for identification of the best combinations to use in future assays. This is especially important for ACh, as only 6 concentrations fit in the multiwell plate used for blood incubation. As inhibition of pro-inflammatory cytokines was most dramatic with the highest ACh concentrations, the lower concentrations were deemed unnecessary or expendable. Therefore, in addition to the zero well containing no ACh, it was decided to continue only with 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M ACh. Out of the three tested PYR conditions, yesPYR (consisting of 1mM PYR) proved the most successful—hiPYR was generally too stimulative, while also overly suppressive of IL-10 (Figure 1b), and noPYR was not stimulative enough, especially in the case of IL-1β (Figure 1d). Thus, the next assays would use these suggested ACh and PYR concentrations.

Experiment 4. ACh Sensitivity to Acute Stress

This experiment investigated whether exposure to acute stress could affect the regulation of inflammatory cytokines by ACh. Though the literature has shown the cholinergic pathway to be relevant for inflammation, differences in ACh sensitivity before and after stress would implicate this pathway as a novel stress system.

4.1 Methods

4.1.1 Participants

Four healthy young adults, consisting of one female and three males, participated in this acute stress experiment. They were recruited by personal communication over the phone and e-mail, fliers around the Brandeis University campus, and a message to all members of the Psychology Club. In addition to the exclusion criteria of Experiments 1 and 2 (see Appendix), participants were screened for smoking behavior and oral contraceptives, which may have an effect on cytokine measurement. Participants were compensated $10 an hour for their commitment to the experiment.

4.1.2 Protocol

Experimentation took place in the mid- to late afternoon to control for time of day. Upon arrival of the participant, the experimenter explained the study protocol and informed consent form. After the participant gave his consent, a venous catheter (Saf-T-Intima IV catheter, BD) was inserted by a registered nurse. Placement of the catheter was followed by a 30-minute rest period to give participants time to recover from the potential stress of the catheter placement or traveling to the laboratory. Then, participants were exposed to the TSST, an established acute stress paradigm (Kirschbaum, et al., 1993). Blood samples were collected in 10.0mL sodium heparin-coated vacutainers at three time points: directly before stress (-1 minute), and at +1 and +50 minutes after stress.

4.1.3 Acute Stress Exposure

The Trier Social Stress Test (TSST) is a standardized method for inducing acute psychosocial stress in study participants. Stress response systems were activated by a situation in which participants were asked to promote themselves in a mock-interview, speaking freely about their personality to a non-responsive couple of authoritative panelists, followed by a difficult arithmetic task (Kirschbaum, et al., 1993). Participants were told that they would be recorded and videotaped, and a microphone and video camera were set in front of them. They were given five minutes to prepare their speech using pen and paper, and they were told they will not be able to use these notes during the interview. Five minutes each were then attributed to the speaking and math tasks. Throughout the 15 minute session, the two panelists held a neutral disposition and maintained eye contact without encouraging the participant.

4.1.4 Blood Processing and Measurement of Cytokine Concentrations

Blood samples were processed immediately according to the suggested procedure determined by Experiment 3. Briefly, whole blood was diluted 10:2 in saline, then 400µL diluted blood was added to wells containing 50µL 100 ng/mL LPS and 50µL ACh of varying concentrations (0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, or 10^{-2} M ACh). After five minutes on a mixer, plates were incubated for 18 hours at 37^{o}C, then centrifuged at 4^{o}C and 2000*g* for 10 minutes. Plasma supernatant from each participant was transferred into an individual storage plate that was stored at -80^{o}C. Cytokine concentrations were measured by ELISA as described in Experiments 2 and 3, with the exception of only using 25µL plasma in the 1:10 dilution for IL-10 (see Table 2).

4.1.5 Statistical Analysis

Data were analyzed using PASW statistics and PRISM 5 software packages. First, effects of ACh concentration and time after stress on cytokine concentration were tested for by repeated measures ANOVAs. In order to see whether stress influenced cytokine concentrations independent of ACh, the cytokine levels derived from samples that had been incubated with 0 M ACh (e.g. saline) were included in cytokine-specific repeated measures ANOVAs. To further test for an effect of stress, absolute inhibition (AI) by ACh was calculated for each cytokine concentration at each time point for each participant. AI was defined as the difference in concentration between a sample treated with some concentration of ACh and the sample treated only with LPS from the same time point. Repeated measures ANOVAs for each cytokine were run for each AI, with time after stress as a within-subjects variable and cytokine concentration as the dependent variable. Fourth, IC_{50} measurements were calculated using PRISM software to generate a one-number ACh sensitivity for each participant at each time point. Finally, these IC_{50}'s for each participant were run in repeated measures ANOVAs to see if stress affected ACh sensitivity. Gender differences were not considered due to there being a single female participant, and all significance values were computed under Greenhouse-Geisser specifications.

4.2 Results

4.2.1 Test for Effects of ACh and Time After Stress

Repeated measures ANOVAs for each cytokine, with ACh concentration and time after stress as within-subjects variables, showed a significant effect of ACh on plasma

TNF and IL-6 but not IL-1β or IL-10 (Table 4). TNF and IL-6 were observed to be dose-dependently inhibited by ACh (Figure 3). Time after stress did not affect cytokine levels, either alone or in interaction with ACh. Thus, determination of the effect of stress required statistical tests with greater specificity.

	TNF		**IL-1β**		**IL-6**		**IL-10**	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
ACh	76.68	< 0.001	1.07	0.38	54.14	< 0.001	5.01	0.10
Time After Stress	0.219	0.70	2.34	0.22	1.37	0.33	0.075	0.83
ACh*Time After Stress	0.602	0.57	0.433	0.43	1.130	0.38	0.77	0.44

Table 4. Cytokine concentrations were partly mediated by ACh but unaffected by stress. ACh significantly inhibited TNF and IL-6, but not IL-1β or IL-10. Cytokine concentrations did not significantly differ before and after stress, nor were any interactive effects of ACh and time after stress observed.

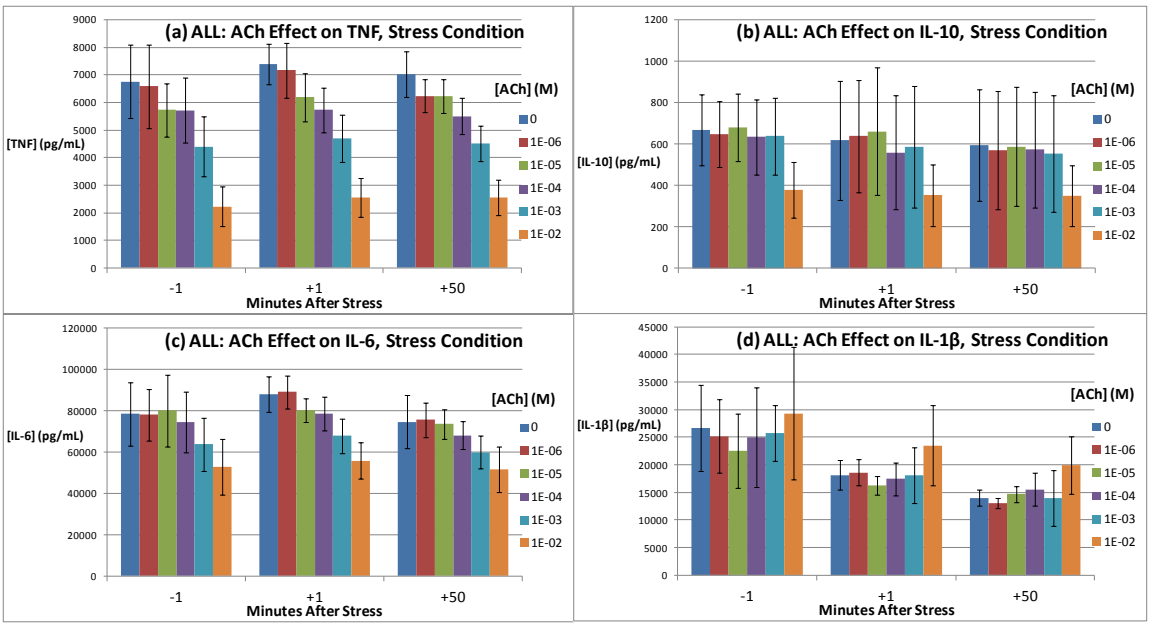

Figure 3. ACh dose-dependently inhibited plasma TNF and IL-6, and acute stress had no effect (n = 4 participants). Incubation of blood with increasing concentrations of ACh resulted in suppression of TNF and IL-6, but did not significantly affect IL-10 or IL-1β. Error bars are standard errors of the mean.

4.2.2 Test for Effect of Stress

Comparison of samples incubated without ACh across time points revealed no differences in TNF, IL-6, or IL-1β; though IL-10 concentrations were significantly different before and after stress ($F = 8.17$, $p = 0.043$). Specifically, IL-10 levels rose from -1 to +1 and partially returned to initial levels at +50 minutes after stress.

4.2.3 Absolute Inhibition (AI) ANOVAs

No significant differences were found between AI concentrations across time points for any cytokine (data not shown), suggesting stress did not affect cytokine levels. This was not surprising, as very large effects would need to be seen in order to reach

significance in this small sample size. However, qualitative analysis of AIs revealed the directional effect of stress on cytokine concentrations. For each cytokine, the ACh concentration that exhibited intermediate inhibition was considered representative and was subjected to further analysis. Though ANOVAs comparing cytokine levels across time points were run for all AIs, these representative AIs proved useful for qualitatively describing the effect of stress on each cytokine. Notably, acute stress caused increased inhibition by ACh in TNF and IL-6 and the reverse effect for IL-10 and IL-1β (Figure 4).

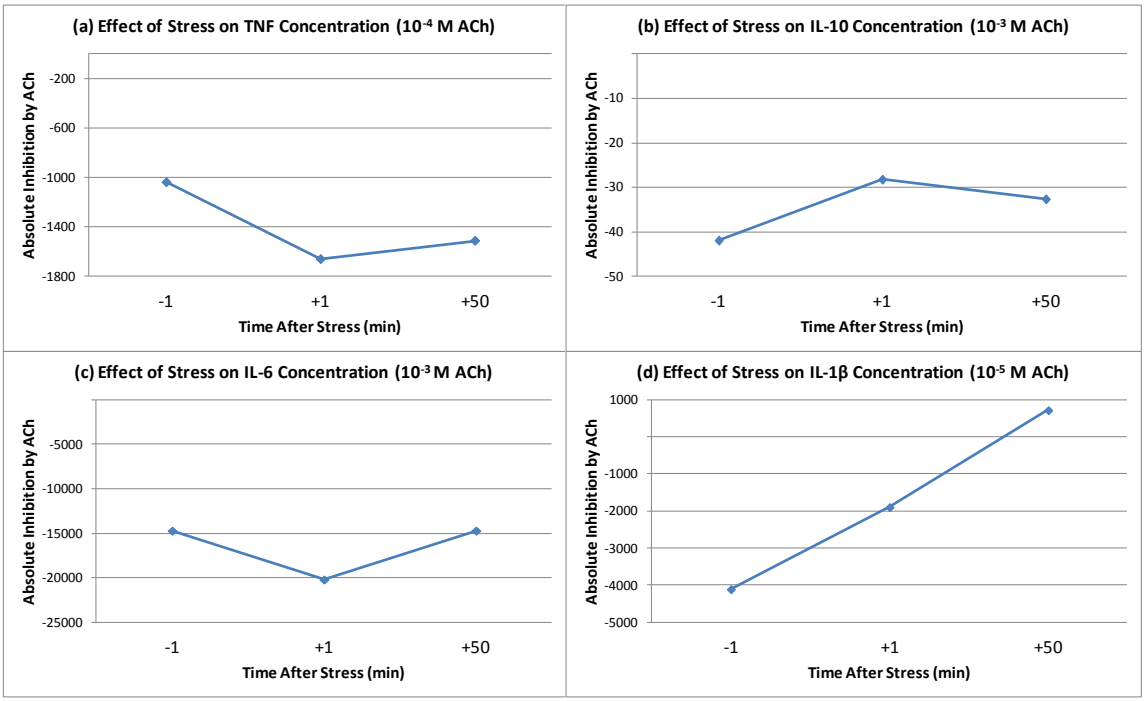

Figure 4. Representative absolute inhibition (AI) values revealed the directional effects of stress on cytokine production over time. Acute stress led to increased inhibition by ACh in TNF **(a)** and IL-6 **(c)**, which partially or fully remediated after 50 minutes. In contrast, stress led to a reduced ACh inhibition of IL-10 **(b)** and IL-1β **(d)**.

4.2.4 IC_{50} Measurements and ACh Sensitivity to Acute Stress

The raw cytokine concentrations were entered into PRISM software to produce an IC_{50} for each participant at each time point for each of the four cytokines. This IC_{50} indicated the necessary ACh concentration to inhibit a given cytokine by 50%, meaning that it could serve as a one-number index of ACh sensitivity. PRISM was also used to produce graphs comparing the IC_{50} values across time points for each participant (Figure 5). The IC_{50} values were run in repeated measures ANOVAs for each cytokine, with time after stress as the within-subjects variable. Acute stress was not found to significantly affect ACh sensitivity of any cytokine in this set of participants.

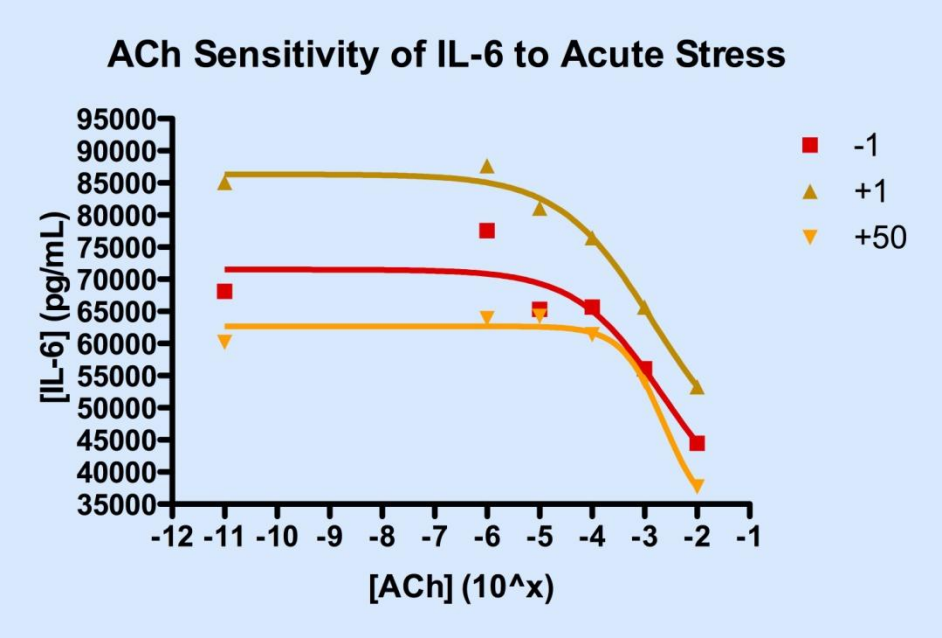

Figure 5. ACh sensitivity of IL-6 to acute stress in one participant. In this example, the curves clearly diverge, indicating a difference in ACh sensitivity at the different times relative to stress exposure.

4.3 Conclusions

Analysis of blood taken at -1, +1, and +50 minutes after acute stress exposure in four young adults revealed no significant effect of stress. Acute stress did not modulate ACh-independent production of cytokines, with the exception of IL-10, and neither representative AI values nor IC_{50}'s showed any significant stress effects. However, all analyses were conducted including data from all four participants. Inspection of individual responses before and after stress uncovered robust effects of stress on cytokine production in some but not all participants, which may have masked changes in sensitivity. The variability of strong stress responses even counter-acted one another in some cases (Figure 6). It is clear that four participants comprised an insufficient sample size for determination of stress effects, as the effect would have needed to be very large for detection.

Though ACh sensitivity to acute stress was not definitively demonstrated, the actions of stress proposed by representative AIs make theoretical sense. Specifically, these AIs suggested that stress reduced inhibition by ACh for TNF and IL-6, and increased inhibition by ACh for IL-10 and IL-1β (Figure 4). Activation of the HPA axis due to the TSST would increase cortisol levels, and glucocorticoids are known to inhibit pro-inflammatory cytokines as well as stimulate the anti-inflammatory IL-10 *in vitro*.

This experiment also confirmed the ACh suppression of pro-inflammatory cytokines TNF and IL-6, as suggested by Experiments 2 and 3 in non-stressed individuals. Additionally, the counterintuitive finding in Experiment 3 that ACh also inhibited IL-10 was somewhat reproduced. ACh did inhibit IL-10 across time points

(Figure 3b), though the large standard error and small sample size contributed to making this effect statistically insignificant, (p = 0.10).

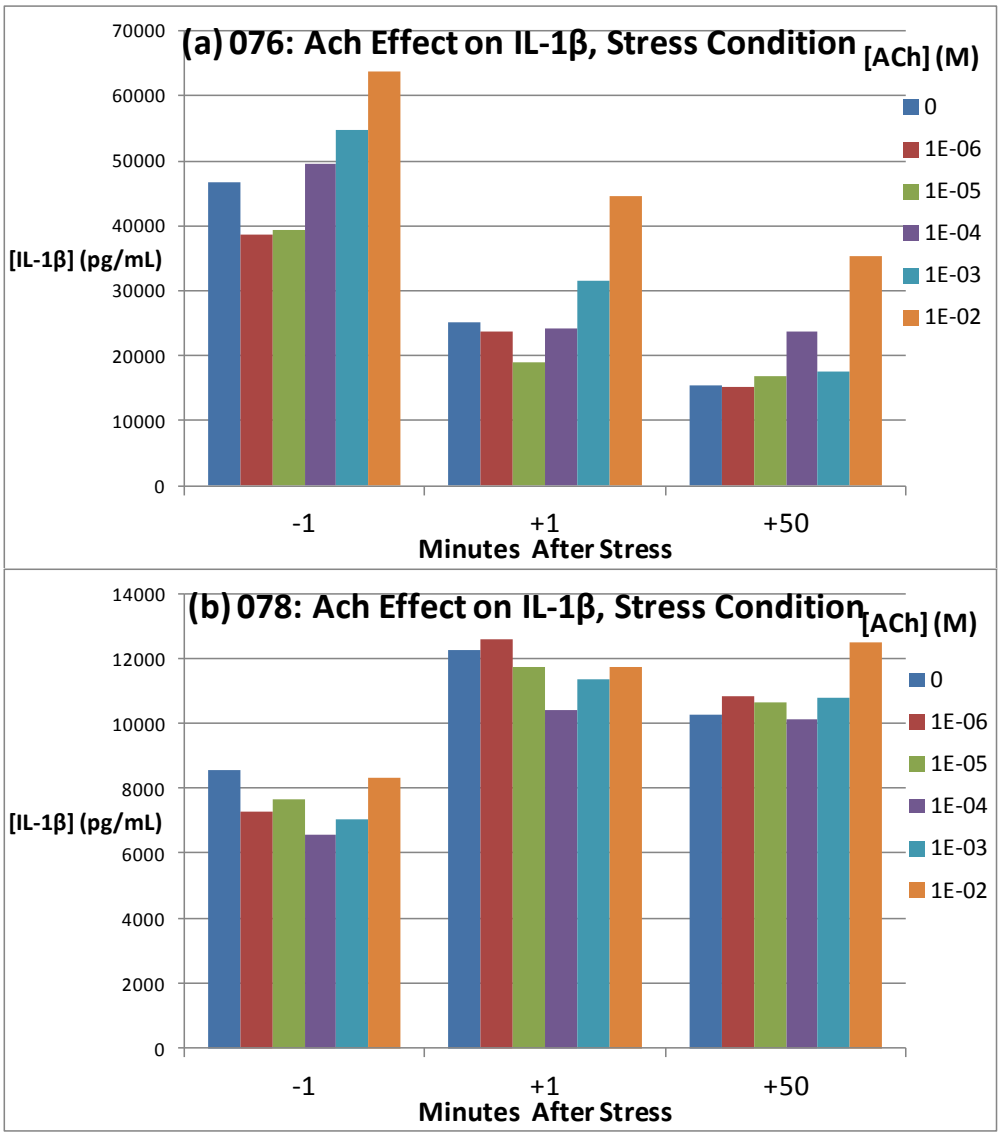

Figure 6. Individual differences in the response to stress may have masked any overall effect. In this example, two participants showed strong effects of stress, but in opposite directions. One participant displayed reduced levels of IL-6 **(a)**, while another responded with increased levels of IL-6 **(b)**. Larger sample sizes are needed to gain greater specificity of ACh sensitivity to stress.

Chapter 3. Discussion

1. Findings

These experiments were conducted to reaffirm the anti-inflammatory effect of ACh and investigate how acute stress affects ACh sensitivity. Experiment 1 successfully determined the necessary plasma dilutions for measurement of inflammatory cytokines in LPS-stimulated blood treated with ACh. Experiment 2 used this setup to measure the effect of ACh on levels of TNF, IL-10, IL-6, and IL-1β, in a group of non-stressed young adults. Though effects were not significant, empirical results suggested ACh inhibition of TNF and IL-6. In order to see the influence of ACh more clearly, the assay was adjusted to include higher concentrations of ACh and PYR in Experiment 3. Here, ACh significantly suppressed plasma TNF, IL-6, and IL-10. PYR was observed to interact with ACh in modulating cytokine levels, but it also did so independent of ACh. This novel finding could mean that the action of PYR is not limited to inhibiting AChE. Optimal concentrations of ACh and PYR for future assays were determined. Experiment 4 utilized this updated blood processing protocol to measure cytokine concentrations before and after stress. Acute stress exposure did not affect cytokine concentrations, but ACh was found to dose-dependently inhibit TNF and IL-6. Taken together, these experiments provide strong evidence that ACh suppresses inflammation through dose-dependent inhibition of TNF and IL-6 levels, consistent with earlier findings in mice (Borovikova et al., 2000).

2. Limitations

Though stress was not found to alter ACh sensitivity in the overall group, inspection of individual data revealed unique stress responses that, when averaged, could have masked the effect of stress (Figure 5). In general, high inter-individual differences in stress response have been observed with exposure to the TSST, so a much larger sample size is needed to detect the effects of stress. Another limitation was the number of blood draws analyzed per participant. Using the provided lab resources, only blood at 3 time points (-1, +1, +50 minutes after stress) was available for measurement. A more complete picture of the stress response and its downregulation would be provided with blood from additional time points (such as +10 and +120 minutes after stress). A third imperfection to these experiments was that the highest ACh concentration (10^{-2} M) was outside the physiological range. Inclusion of 10^{-2} M ACh may therefore have skewed the statistical effect of ACh, considering this concentration exhibited the greatest inhibitory effects.

3. Future Directions

Importantly, this research provides the first characterization of the cholinergic anti-inflammatory pathway with respect to its sensitivity to psychosocial stress. In comparison, acute stress-induced changes in inflammatory sensitivity of glucocorticoids, as an element of the HPA axis, have been studied in detail over the past twenty years (reviewed in Steptoe et al., 2007). Despite there being no significant stress effects on ACh sensitivity, representative absolute inhibition values for each cytokine did show qualitative differences (Figure 4). Therefore, it is promising that future studies, with

greater sample sizes, will detect changes in ACh sensitivity due to acute stress. Once this result is achieved, ACh sensitivity to stress can be compared to glucocorticoid and catecholamine responsivities so as to provide a relative index of how each of the corresponding stress systems (PNS, HPA axis, and SNS, respectively) regulates inflammation. Individual ACh sensitivities to stress should also be related to physiological risk factors for disease, such as body-mass-index (BMI) and percent body fat. Obesity is associated with increased prevalence of a number of diseases, including type 2 diabetes, gallbladder disease, and coronary heart disease (Must et al., 1999). If changes in ACh sensitivity are shown to be correlated with BMI and percent body fat, it could indicate a role for the PNS in this obesity-disease association.

4. Implications

The main finding that ACh dose-dependently inhibited the pro-inflammatory TNF and IL-6 in humans confirms the cholinergic pathway as anti-inflammatory. This evidence that the PNS has immunoregulatory functions has major implications for therapeutic strategies. Ultimately, this line of research will direct development of protective treatments that target the cholinergic pathway to prevent excessive inflammation and associated diseases. Recent research has already begun to focus on this pathway. For instance, drug activation of the α7 nicotinic ACh receptor can reduce the risk of ischemia-related cell death (Norman et al., 2011). Evidence that electrical stimulation of the vagus nerve can act to suppress immunity (Borovikova, et al., 2000; Czura, et al., 2010) also implicates the vagus nerve as a potential target. In the future, nerve stimulators may thus replace drugs that have targeted cytokine production, in order

to more directly attenuate inflammation (Huston & Tracey, 2011). In short, the present research has improved characterization of the cholinergic regulation of inflammation, highlighting this pathway as a therapeutic target. Therefore, this cholinergic anti-inflammatory pathway is immediately relevant for improvement of our ability to treat or prevent disease outcomes.

APPENDIX

SCREENING PROTOCOL

1) BMI: between 18 and 35.
If no, exclusion.
2) MENSTRUAL CYCLE (Females Only): Do you have a regular menstrual cycle?
If no, exclusion.
3) BLOOD: Did you receive vaccinations or donate blood in the past 2 weeks?
If yes, schedule at least 2 weeks after.
4) NEEDLES: Does having IVs inserted or having blood drawn cause you a lot of anxiety, fear, or pain?
If yes, exclusion.
5) VEINS: Have you been told that your veins are hard to find? Does it always take more than one try to find a vein when giving or having a blood draw?
If yes, exclusion.
6) STRESS: Extraordinary stress at present?
If yes, exclusion.
7) TSST: Have you participated in any studies from the Brandeis Health Psychology Lab?
Exclusion if have done TSST (Trier Social Stress Test) in past.
8) Do you have any atopic diseases? (ex. asthma, atopic dermatitis)
If yes, exclusion.
9) Do you have any allergies? (ex. to pollen, latex) If so, do you take any seasonal medications?
If yes, exclusion.
10) Do you have any current autoimmune disorders? (ex. rheumatoid arthritis, multiple sclerosis, lupus)
If yes, exclusion.
11) Do you have any infectious diseases? (ex. HIV, hepatitis, tuberculosis)
If yes, exclusion.
12) Do you have any thyroid diseases?
If yes, exclusion.
13) Do you have any cardiovascular diseases?
If yes, exclusion.
14) Do you have any psychiatric diseases? (ex. depression, anxiety, or other diagnosed illnesses)
If yes, exclusion.
15) Do you have any diseases of the airway currently? (ex. pneumonia)
If yes, exclusion.
16) Do you have any diseases of the urinary tract?
If yes, exclusion.
17) Do you have any diseases of the gastrointestinal system?
If yes, exclusion.
18) Do you have diabetes?
Exclusion if type 1 or type 2.
19) Other chronic diseases?
20) Please indicate any regular prescribed medication.
Checked with thesis advisor, Nic Rohleder, if any prescribed medications.

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