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**Altered Human Neutrophil Function in Response to Acute Psychological Stress**

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**Objective:** To examine the effects of an acute laboratory psychological stress task on neutrophil function, specifically phagocytosis of Escherichia coli and stimulated superoxide production in human neutrophils. There is mounting evidence that acute stress is associated with short-term increases in a number of immune indices.

**Methods:** Participants were 40 (n = 20 females) university students (mean age, 25.9 ± 4.56 years). Blood samples to determine neutrophil function by flow cytometry were taken at the end of resting baseline, during an acute stress task, and during recovery. The stress task was a 10-minute time-pressured mental arithmetic challenge with social evaluation.

**Results:** There was an acute increase in phagocytic ability, $p = .047$, $\eta^2 p = 0.076$, and a reduction of superoxide production, $p = .026$, $\eta^2 p = 0.101$, associated with the stress task relative to baseline.

**Conclusion:** These findings suggest that neutrophil bactericidal function may be sensitive to mental challenge tasks that provoke acute psychological stress. Further research is needed to replicate the observed psychological stress-induced changes in neutrophil function.

**Key words:** acute psychological stress, neutrophil, phagocytosis, superoxide production.

**SBP** = systolic blood pressure; **DBP** = diastolic blood pressure; **HR** = heart rate; **MFI** = mean fluorescent intensity; **EDTA** = ethylene diamine tetraacetic acid.

**INTRODUCTION**

Acute and chronic psychological stress seem to have a differential impact on the immune system. Generally, chronic stress has been shown to have a negative effect on various aspects of immunity, whereas acute stress would seem to be immune enhancing (1). For example, acute stress has been shown to elicit lymphocytosis (2,3), increase natural killer cell cytotoxicity (4,5) and secretory immunoglobulin A secretion rate (6), stimulate aspects of the complement system (7), and boost vaccination responses (8). However, little is known about the effects of acute stress on human neutrophils and particularly their bactericidal function. This is perhaps surprising, given that neutrophils are a major component of innate immunity and are the dominant leukocyte in the circulation. These cells play a crucial role in killing invading pathogens, particularly rapidly dividing bacteria, and are key cellular components of the early phase of inflammatory responses (9). Neutrophils act quickly and without specificity and have the ability to produce a range of cytotoxic and bactericidal molecules, such as reactive oxygen species (superoxide production) and proteolytic

enzymes (10). Superoxide production is a means by which neutrophils eliminate pathogens and, thus, is key in combating infections by, for example, pneumococcal bacteria (11,12). However, extended neutrophil activation and superoxide production can cause local tissue inflammation and damage (13,14). Furthermore, tissue damage via neutrophil activation is implicated in the etiology and aggravation of chronic inflammatory disease (9,14). Finally, more recent research (9,15) suggested that neutrophils may also play a role in acquired immunity by acting as antigen presenting cells.

The effect of stress on neutrophils has been examined in a series of studies of asthma (16 – 18). Academic examinations served as the source of psychological stress, and neutrophil function was assessed before, during, and after an examination period, using the superoxide dismutase-inhibitable reduction of ferricytochrome C assay to measure superoxide release. These authors found some indication that examination stress increased superoxide release. However, it has been argued that a prolonged examination period constitutes chronic rather than acute stress; whereas a single examination elicited an increase in secretory immunoglobulin A, a prolonged period of academic examinations was associated with a decrease (19). More recently, a 15-minute laboratory stress task (Raven's progressive matrices) was used to examine neutrophil activation, using the nitro-blue tetrazolium assay that indicates oxidative capacity (20). The acute stress was shown to increase neutrophil activation relative to baseline. However, neither of these assays are optimally sensitive measures of neutrophil function. The superoxide dismutase-inhibitable reduction of ferricytochrome C assay only detects superoxide release that has escaped from the cell into the medium, which may represent only a fraction of the superoxide actually generated (21). The nitro-blue tetrazolium assay measures, by microscopic inspection of staining, the percentage of nonspecifically activated neutrophils (20). Both of these measures represent a very imperfect proxy of superoxide release. In contrast, the new generation of assays that exploit flow cytometry technology to measure the effects of stimulation accordingly offer a more accurate and versatile means of examining different aspects of neutrophil function. These include phagocytosis and superoxide production in response to a range of stimuli (22–24). In addition, physiologically relevant agents, such as opsonized bacteria, can be used to elicit the neutrophil response.

Consequently, the present study examined the effects of an acute laboratory psychological stress task on neutrophil function, specifically phagocytosis and stimulated superoxide production, using this new generation of assays. Self-report and cardiovascular measurements were also taken as a manipulation check, as the effects of the stress task on these variables are well characterized (3,25). It was hypothesized that acute stress would enhance neutrophil function relative to baseline.

**METHODS**

**Participants**

Forty healthy participants (n = 20 females, 20 males) were recruited from the student population at the University of Birmingham between May 2008 and June 2009. Date of birth, sex, ethnicity, parental occupation, health, and smoking status were determined by standard questionnaire. Participants' mean (standard deviation) age was 25.9 (4.56) years and mean body mass index was 23.3 (2.53) kg/m2. The majority (90%) reported their ethnicity as white and that

they were nonsmokers, and 74% reported that they came from nonmanual occupational households. Individuals were excluded if they had an acute infection or suffered from an immune disorder.

### Design and Procedure

The design was a within-subjects ABB design with baseline, stress, and recovery measurements in each participant. All participants attended a 2-hour laboratory acute psychological stress session. They were asked to avoid alcohol and vigorous exercise for 12 hours, and food and caffeine for 1 hour before testing. On the testing day, participants entered the laboratory at 9.30 AM. Their height and weight were measured. An intravenous cannula was then inserted in the antecubital region of one arm, and a blood pressure cuff was attached to the other arm. Post instrumentation and laboratory adaptation, there was a baseline resting period of 20 minutes, a 10-minute psychological stress task, and a10-minute resting recovery period.

The study was approved by the local Medical Research Ethics Committee, and all participants provided their written informed consent.

### Acute Stress Task

Participants undertook an acute psychological mental stress task, the Paced Auditory Serial Addition Test, which has been shown to elicit cardiovascular changes in many studies (3,7,25). Participants were presented with a series of single-digit numbers by audio-CD and requested to add each number to the immediately preceding number and say the answer aloud at the same time retaining the second of the pair in memory for addition to the next number presented. The 10-minute task consisted of five consecutive and continuous periods of 2 minutes, with numbers presented at speeds of 50, 65, 75, 100, and 125 digits at presentation rates of 2.4, 2.0, 1.6, 1.2, and 0.8 seconds, respectively. All participants were given an explanation of the task and an opportunity to practice before its commencement. To add to the challenging nature of the task, elements of competition and social evaluation were added. The social evaluation was included to increase the provocative nature of the task (26). A false leader board was in view of the participant, who was instructed to try to beat the scores on the board. A confederate experimenter scored the answers overtly at the same time sitting on a high stool at a distance of 1 meter facing the participant. Participants were seated in front of a large television screen, which allowed them to see themselves live throughout the test, and they were instructed to look at the screen at all times. They were also informed that they were being videotaped and that videos were to be assessed by "independent body language experts"; no such assessment was made. The confederate experimenter also sounded a loud aversive buzzer once during the first five of every ten trials. Participants began the test with a score of 1000 points; for each incorrect or missed answer, 5 points were deducted from their score. Immediately after the task, they rated how stressful, difficult, and engaging the task was on a 7-point scale, where 0 equaled "not at all" and 6 equaled "extremely."

### Blood Sampling and Immune Assays

Blood samples were taken at three time points: at the end of the resting baseline; in the final minute of the stress task; and at the end of the recovery period. At each time point, a blood

sample was collected into one ethylenediaminetetraacetic acid tube and one heparin tube, which was placed immediately on ice. The ethylenediaminetetraacetic acid tubes were used to count lymphocyte and granulocyte numbers, using a Coulter A ● T diff analyzer (Beckman Coulter, Inc., Miami, Florida). The heparin tubes were used to assess neutrophil function in whole blood, specifically bacteria-induced phagocytosis and oxidative burst (superoxide production) by flow cytometry.

Phagotest kits (Orpegen Pharma GmbH, Heidelberg, Germany) measured neutrophil phagocytosis of fluorescein isothiocyanate-labeled opsonised *Escherichia coli* (*E. coli*) bacteria. Whole blood (100 μL) was incubated with 20 μL of bacteria (2 x $10^7$) at 37°C for 10 minutes, whereas a negative control sample remained on ice. For further details, see the manufacturer's test protocol (http://www.icp.ucl.ac.be/mexp/phagotst.pdf). Using a three-laser- Dako Cyan High Performance flow cytometer (Dako, Carpinteria, California) with Summit v 4.3 software, neutrophils, among other granulocytes, were gated on forward and side scatter characteristics; the mean fluorescent intensity (MFI) corresponding to the number of bacteria engulfed by a single cell was measured, using a blue-green excitation light 488 nm argon-ion laser.

The Phagoburst test (Orpegen Pharma GmbH) determined the mean fluorescence per neutrophil from the production of reactive oxidants with or without stimulation. In summary, four tubes contained 100 μL of heparinized whole blood that was incubated for 10 minutes with one of the following stimuli: 20 μL of the weak control stimulus; 20 μL of opsonized *E. coli* (2 x $10^7$ bacteria); 20 μL of the strong control stimulus; or without a stimulus at 37°C. The formation of reactive oxidants was monitored by the oxidation of the substrate, from nonfluorescent dihydrorhodamine 123 to rhodamine, which produced green fluorescence, and was analyzed with by cytometry as described above. For further details, see the test protocol (http://www.pvi.uni-bonn.de/bursttst.pdf).

### Cardiovascular Measurements

Blood pressure and heart rate were assessed using a semiautomatic oscillometric blood pressure monitor (Dinamap 1846, Critikon, Tampa, FL, USA) every 2 minutes: in minutes 12, 14, 16, and 18 of the 20-minute baseline period; and minutes 2, 4, 6, and 8 of the stress task period and recovery period.

### Statistical Analyses

To check the psychological impact of the stress task, ratings of stress, difficulty, and engagement were scrutinized. Blood pressure and heart rate measurements were averaged separately across baseline, stress task, and recovery periods and analyzed, using a repeated-measures analysis of variance (baseline, task, recovery), with the Greenhouse-Geisser correction. The same analytic strategy was applied to lymphocyte and granulocyte numbers and to the neutrophil function data. Partial eta-squared ($\eta^2$) was used as a measure of effect size. Slight variations in degrees of freedom reflect occasional missing data.

### RESULTS

### Psychological and Cardiovascular Impact of the Stress Task

The mean (standard deviation) ratings for the Paced Auditory Serial Addition Test were 4.0 (1.19), 4.1 (1.34), and 4.6 (1.17) for stress, difficulty, and engagement, respectively. There was a significant main effect of time, for systolic blood pressure (SBP), $F(2,78) = 119.53$, $p < .001$, $\eta^2 = 0.754$, diastolic blood pressure (DBP), $F(2,78) = 52.05$, $p < .001$, $\eta^2 = 0.572$, and heart rate (HR), $F(2,78) = 67.83$, $p < .001$, $\eta^2 = 0.635$. These effects are shown in Figure 1.

### Lymphocyte and Granulocyte Numbers

The summary lymphocyte and granulocyte number data are also presented in Figure 1. For lymphocytes, there was a significant main effect of time, $F(2,78) = 14.21$, $p < .001$, $\eta^2 = 0.267$. For granulocytes, there was also a significant main effect of time, $F(2,78) = 10.22$, $p < .001$, $\eta^2 = 0.208$. In both cases, it is clear that the acute stress was associated with a transient increase in circulating leukocytes, which decreased after cessation of the task.

### Neutrophil Function

For neutrophil phagocytosis measurements, all samples showed that 100% of the neutrophils were able to phagocytose the *E. coli*, and differences seen were in the number of bacteria ingested (indicated by differences in MFI). Stimulated and unstimulated immune fluorescent intensities at the three time points are shown in Table 1. There was a significant main effect of time on neutrophil phagocytic capacity, $F(2,78) = 3.20$, $p = .047$, $\eta^2 = 0.076$, as shown in Figure 2. Thus, the stress task seemed to have enhanced phagocytosis. For superoxide production, when granulocytes were stimulated by *E.coli*, the opposite pattern of effects emerged. There was a significant reduction in superoxide production across the session, $F(2,72) = 4.03$, $p = .026$, $\eta^2 = 0.101$, as illustrated in Figure 2.

Neutrophil reactivity was not associated with gender (women: mean, 6.30; men: mean, 5.66, $p = .91$, for phagotest; and women: mean, -10.40; men: mean, -1.46, $p = .41$, for phagoburst to *E. coli*), body mass index ($r = .16$, $p = .33$, for phagotest, and $r = .02$, $p = .88$, for the phagoburst to *E. coli*), or smoking status (current smokers: mean, 8.37; nonsmokers: mean, 5.71, $p = 79$). Baseline neutrophil function was not related to neutrophil reactivity with the exception of superoxide production to *E. coli*, where there was a negative association and baseline accounted for 27% of the variation in neutrophil reactivity, $r = -.48$, $p = .003$.

### Sensitivity Analysis

Additional analyses were run, using phagotest and phagoburst values with the subtraction of the unstimulated MFIs in order to examine any influence of psychological stress on unstimulated neutrophil function. For phagocytosis, the main effect of time was attenuated, $F(2,74) = 1.98$, $p = .14$, $\eta^2 = 0.51$. For phagoburst to *E. coli*, the significant time effect remained, $F(2,72) = 4.03$, $p = .026$, $\eta^2 = 0.101$. However, there were no significant main effects of time on neutrophil function in the control/unstimulated tubes.

### Association Between Cardiovascular and Neutrophil

**Activity During Acute Stress**

There were no significant correlations between cardiovascular activity during stress and neutrophil function: SBP: $r$ = -.05, $p$ = .75; DBP: $r$ = .06, $p$ = .67; HR: $r$ = -.03, $p$ = .83 for phagotest; and SBP: $r$ = .19, $p$ = .24; DBP: $r$ = .22, $p$ = .17; HR: $r$ = -.007, $p$ = .96 for phagoburst to *E. coli.*

**DISCUSSION**

The self-report and cardiovascular data confirmed that the acute stress task provoked emotional and physiological responses. Similarly, as expected, there was an acute stress induced increase in lymphocyte and granulocyte numbers. The effects of stress on neutrophil function, however, were more complex. For neutrophil phagocytosis, there was an acute increase in phagocytosis associated with the stress task. This was attenuated in analyses where the MFI of the control tubes are incorporated into the dependent variables, but this could reflect a reduction in degrees of freedom, as there was no significant effect of time on phagocytic ability in the control/ unstimulated tubes. However, the acute stress task was associated with reduction in neutrophil superoxide production when stimulated with *E. coli*.

Given the preliminary nature of this study, further research is needed to replicate the observed psychological stress-induced changes in neutrophil function. However, the present study is the first we know of to examine the effects of psychological stress in humans, using the new generation of assays that measure neutrophil function via flow cytometry techniques and in response to physiologically relevant stimuli. Accordingly, the existing literature offers little help in establishing a precedent for our findings. However, the effects of the acute stress task on neutrophil phagocytosis observed here are consistent with the increase in neutrophil activation to a short mental stressor reported previously (20). Superoxide production in the present study decreased in contrast to the increase with examination stress shown in previous reports (17,18). However, it should be noted that academic examination stress over several weeks cannot be considered to be acute psychological stress. Furthermore, studies evaluating neutrophil function and regulation have shown that phagocytosis and intracellular bacterial killing are independent activities of neutrophil-mediated antibacterial defense (27,28). Accordingly, it is feasible that phagocytosis and stimulated superoxide production could be differentially affected by acute stress.

Neutrophil function was differentially sensitive to acute psychological stress in the present study. However, cardiovascular activity did not seem to be related to neutrophil function. This is surprising, given that neutrophils have been shown to have α- and β-adrenergic receptors (29,30), and changes in adrenalin and noradrenalin have been shown to relate to circulating neutrophil numbers (30). However, it is possible that the effects observed in the present study are mediated by other stress pathways, such as the hypothalamic-pituitaryadrenal axis. For example, others (31) have shown in an animal model study that dexamethasone (acting as a cortisollike synthetic stimulant) can significantly decrease neutrophil superoxide production in cats. In vitro studies (32,33) of human neutrophils have also shown that cortisol is able to suppress neutrophil superoxide generation directly. Consequently, the declining superoxide production to *E. coli* observed here might relate to the effects of the acute stress task on cortisol levels, although measurements of serum cortisol would be needed to confirm this. It is difficult to elucidate the exact mechanisms behind the

different phagocytosis and superoxide production results observed here, given that the regulation of neutrophil function is under the control of many different receptors for a variety of cytokines (27), which are also differentially affected by stress (34,35).

The present study might be considered to have some limitations. First, the sample size was fairly modest. However, it was of the same order of magnitude as previous studies of stress and human neutrophil function (17,20,36) and larger than studies examining the effects of acute psychological stress on other immune outcomes (4,7). Furthermore, study numbers were reasonable, given the temporal demands of the current protocol, with cannulation, time of testing session, and same-day flow cytometry assays. Second, despite the advantages of the flow cytometry assays, they are sensitive to diurnal and day-to-day variation (22,37). However, such effects were minimized by testing at the same time of day and the using internal controls for each assay. Finally, the design would have been stronger with an appropriate no stress control group to unequivocally discount alternative explanations for our findings, such as the effects of cannulation. However, with our pretask baseline, the most parsimonious explanation for the pattern observed is perturbation by acute stress. Neutrophils are the first cells to infiltrate sites of wounding/inflammation; thus, any effect of cannulation would have been evident at baseline, although an additional baseline measure would have confirmed a lack of cannulation effect.

In conclusion, the present study revealed the effects of psychological stress on neutrophil function. The acute stress task elicited a short-term elevation in phagocytosis and a reduction of superoxide production. The present results must be regarded as preliminary, and replication is necessary. Given that neutrophil function has been shown to decline with age (38), future studies might consider the effects of age on the effects of acute psychological stress. In addition, the underlying mechanisms of acute stress effects on neutrophil function warrant further investigation.

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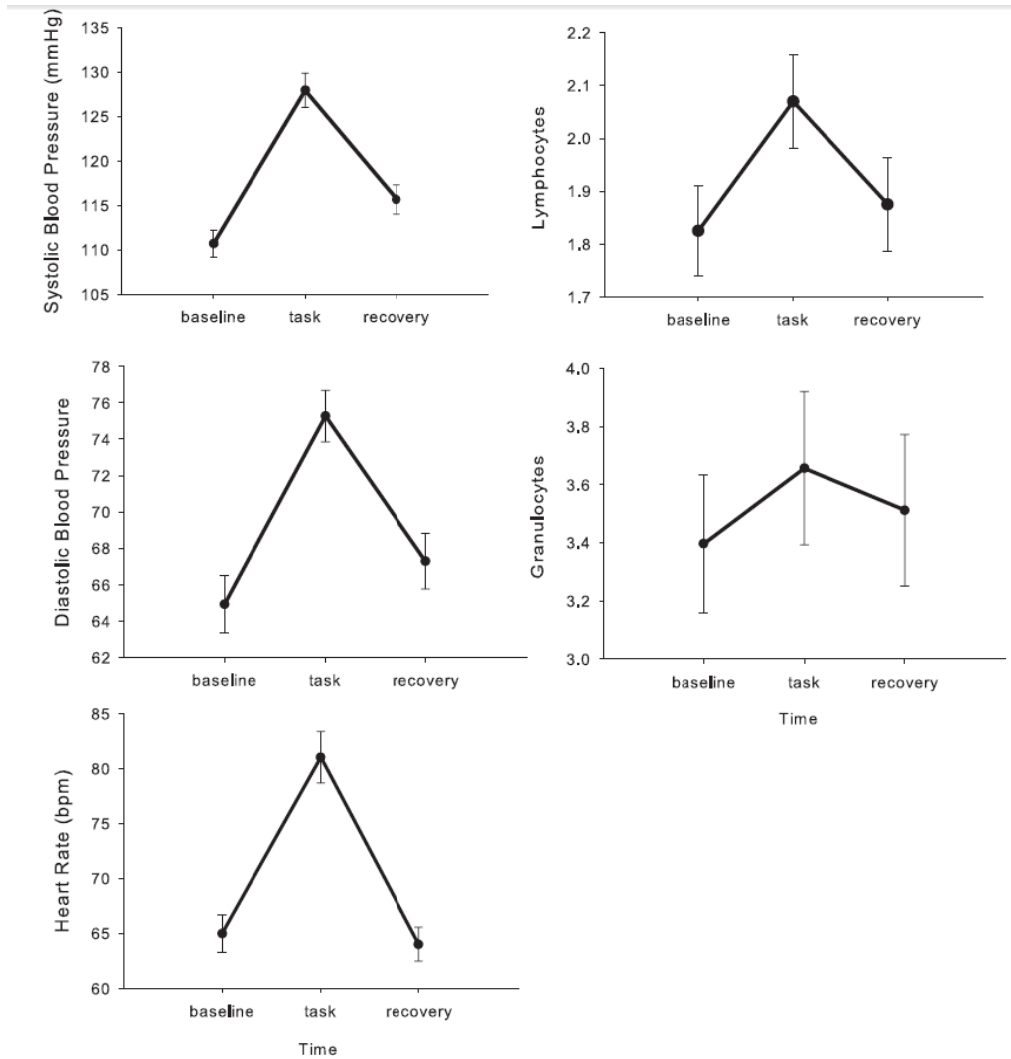
### Tables and Figures



**Figure 1.** Cardiovascular activity and lymphocyte and granulocyte numbers during baseline, stress, and recovery (*error bars* are standard error of the mean).

**TABLE 1. Measurements of Mean Fluorescent Intensities of
Unstimulated and Stimulated Neutrophil at Baseline, Task,
and Recovery**

| Stimulation | Mean (SD) | | |
| --- | --- | --- | --- |
| | Baseline | Task | Recovery |
| Phagocytosis unstimulated | 4.5 (2.18) | 4.8 (2.46) | 4.8 (2.26) |
| Phagocytosis *Escherichia coli* | 143.0 (55.17) | 150.3 (56.68) | 149.9 (59.85) |
| Phagoburst unstimulated | 6.2 (1.57) | 6.1 (1.46) | 6.2 (1.43) |
| Phagoburst *E. coli* | 182.7 (87.40) | 177.2 (75.98) | 169.4 (72.22) |

SD = standard deviation.

**Table 1.** Measurements of Mean Fluorescent Intensities of Unstimulated and Stimulated Neutrophil
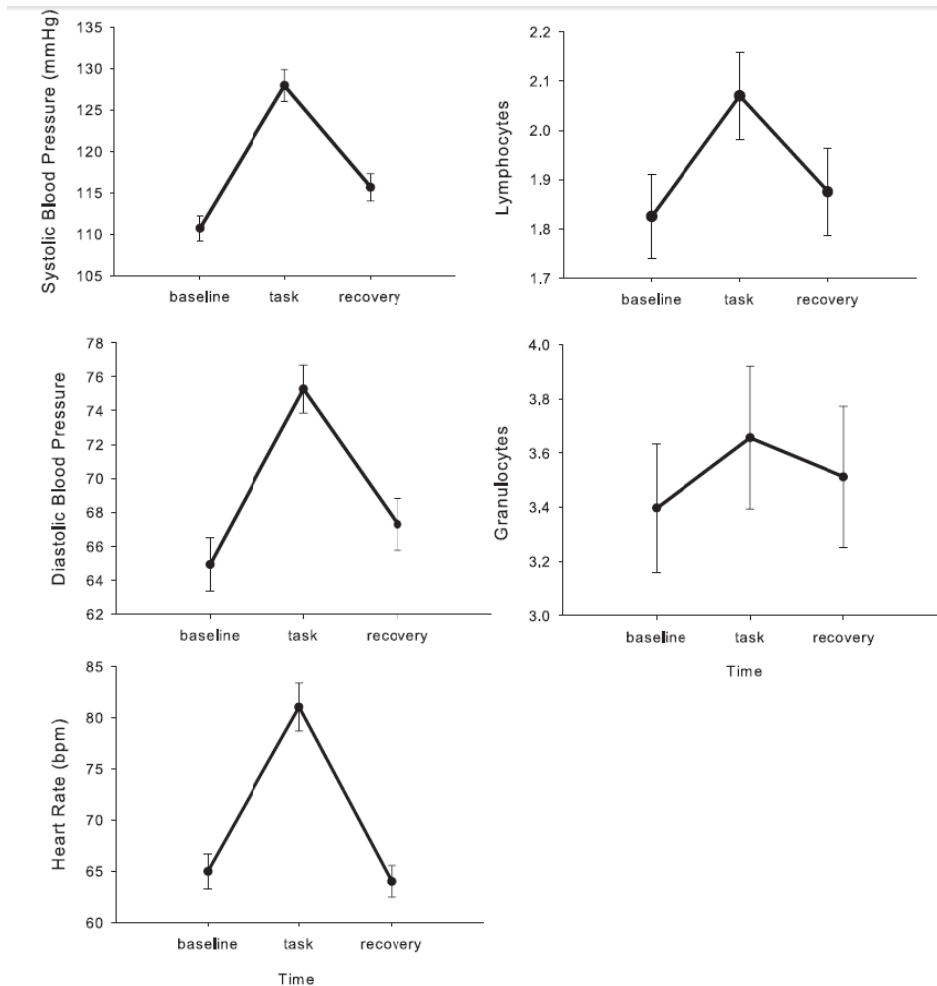at Baseline, Task, and Recovery



**Figure 2**. Neutrophil phagocytosis of *Escherichia coli* and superoxide production on stimulation
with *E. coli* during baseline, stress, and recovery (*error bars* are standard error of the mean).