Study Protocol

Official Title: Transduction of Psychological Stress into Systematic Inflammation by Mitochondrial DNA Signaling (Brief Title: Biological Response to Brief Psychological Challenge)

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Scientific Background

Psychosocial stress is pro-inflammatory and confers increased risk for chronic inflammatory and mental health disorders. However, the mechanism by which stress is transduced into inflammation is not fully understood. Emerging evidence shows that stress can affect the function and integrity of mitochondria – unique bacteria-derived organelles with their own genome – and that "stressed" mitochondria release immunogenic molecules that trigger inflammation systemically. These new results position mitochondria and the release of mitochondria-derived signaling molecules ("mitokines") at the center of the stress-inflammation cascade.

Under conditions of psychosocial stress, mitochondria release mitochondrial DNA (mtDNA), which is a pro-inflammatory molecule. Bacteria-derived mtDNA contains molecular markers that make it particularly immunogenic. In humans, our preliminary work provides initial evidence that acute socio-evaluative laboratory stress is sufficient to induce a robust and selective elevation in serum ccf-mtDNA after 30 minutes. Moreover, in cultured human cells, we find that the stress mediator glucocorticoid promotes mtDNA release from mitochondria. However, the extent to which stress-induced increases in ccf-mtDNA contribute to the delayed increase of circulating proinflammatory mediators after acute psychological stress in humans remains unknown.

In the current study, we proposed a controlled trial to better characterize stress-induced regulation of ccf-mtDNA release and examine its association with the increase in proinflammatory mediators that is known to follow acute psychological stress and though to contribute to physical health risk.

Preliminary work relevant to the Aims of the proposed study: To test whether mtDNA is released in response to psychological stress, we conducted a preliminary uncontrolled laboratory study assessing levels of ccf-mtDNA before and after an acute (5-min) socio-evaluative stressor on two separate occasions. The stressor was chosen for its ability to evoke glucocorticoid hormone release and reliably stimulate increases in circulating markers of inflammation, which typically peak 1-2 hours post-stress. We developed a rigorous approach to quantify serum levels of two mtDNA genes (CYTB and ND1) in 50 healthy midlife individuals (mean age = 50 years, range: 41-58). Serum was collected at baseline (pre) and immediately (post) and 30 minutes after stress (+30 min). DNA was isolated and analyzed by a blinded operator and normalized to a standard curve.

Ccf-mtDNA levels dramatically increased +30 min after the stressor, exhibiting a large effect size ($\eta 2 = .556$, $P < 0.0001$, Figures in Research Plan), with 93% of participants showing an increase in ccf-mtDNA (CYTB). This result was replicated at a second visit, yielding similar results and a comparable effect size. Analyses of the second amplicon ND1 confirmed these findings at both sessions. Findings provide robust initial evidence for psychological stress-induced increases in the highly immunogenic molecule, ccf-mtDNA, but did not establish the time kinetics, the maximal amplitude of this response, or the association of this response with later increases in inflammatory mediators. These are gaps in our knowledge that will be examined in the proposed study.

Both mitochondrial and nuclear genomic fragments can be found in blood. Prior evidence shows that exhaustive physical exercise is followed by increases in both ccf-mtDNA and nuclear-derived ccf-nDNA, possibly due to tissue damage. To ascertain whether psychological stress-induced ccf-mtDNA could result from unspecific cellular damage and non-specific bulk cellular

material release, we also assessed two nuclear gene markers in our preliminary work. In contrast to ccf-mtDNA, ccf-nDNA (GUSB) was unchanged at +30 min on both occasions of testing. Accordingly, at +30 min after stress the ratio of circulating mitochondrial to nuclear genome mtDNA/nDNA rose by >2-fold.

The proposed study will examine physiologic responses to acute psychological challenge in the laboratory among healthy adults. It is widely accepted that there is an increase in circulating markers of inflammation following a single bout of laboratory stress. This increase in systemic inflammation is believed to contribute to the damaging health effect of psychological stress. However, to date the biological mechanisms by which psychological stress is transduced into inflammation are unclear. Our preliminary evidence suggests that mitochondrion may play a role, with stress-induced increases in circulating levels of mitochondria- derived signaling molecules that are known to modulate immune cell function and the production of pro-inflammatory cytokines.

To test this possibility, we plan to conduct a crossover experimental trial examining physiological responses to an evaluative speech task under laboratory conditions. We have previously used this task to induce physiological arousal. We plan to recruit 60 non-smoking volunteers (50% female, aged 20-50 years) and test them on two occasions separated by at least a month. On one occasion they will be exposed to the speech task. On the other occasion, they will rest quietly for the same period. Conditions will be counterbalanced. At both visits cardiovascular responses (heart rate, blood pressure, and heart rate variability) will be assessed as measures of autonomic activation before, during and after the task period. Participants will also have an intravenous catheter inserted and blood drawn at ten time points over the two-hour testing period on each occasion. Blood samples will be sent to laboratories at the University of Pittsburgh and at Columbia University for the assessment of mitochondria-derived signalling molecules, inflammatory markers, cortisol and catecholamine levels.

Study Objectives

Our overarching hypothesis is that psychosocial stress regulates ccf-mtDNA release, which, in turn, signals a proinflammatory response. We examine this hypothesis in parallel human and cell-based studies. The current Clinical Trial only covers Aim 1 of the parent grant, which is the human study.

Specific Aim 1 (human stress study). Determine the temporal relationship between psychological stress, ccf-mtDNA, and systemic inflammation. We hypothesize that socioevaluative stress will trigger robust and transient ccf-mtDNA release compared to a non-stressed condition. To test this, 60 volunteers (50% female; aged 20-50) will be tested on two occasions separated by at least 1 month. On one occasion, they will be exposed to the a socio-evaluative speech stressor known to elicit increases in circulating markers of inflammation at 40-90 minutes. On the other occasion, they will rest quietly for the same period of time. Conditions will be counterbalanced across subjects and blood drawn at ten time points over two hours. We expect that the stress task will induce a specific increase in ccf-mtDNA, which will statistically mediate subsequent peak circulating IL-6 and TNF-α levels. To validate that the stress task was associated with physiological arousal, we will examine task-related changes in heart rate, blood pressure, heart rate variability, and circulating levels of cortisol, epinephrine, and norepinephrine. This study will establish the kinetics and magnitude of psychological stress-induced ccf-mtDNA release, and whether ccf-mtDNA mediates the inflammatory response to acute stress in humans.

Study Design & Methods

We propose a crossover experimental trial examining physiological responses to a socio-evaluative speech task under laboratory conditions. Participants will be tested on two occasions separated by at least 1 month. On one occasion, they will be exposed to the speech task. On the other occasion, they will rest quietly for the same period of time with identical assessment in the absence of the stressor. This control session will allow us to assess and control for any possible effects of the passage of time (e.g., circadian variation in cortisol levels), simply being assessed (e.g., discomfort), and the intravenous catheter. Conditions will be counterbalanced in randomized starting order across subjects and blood drawn at ten time points over two hours to assess ccf-mtDNA, markers of inflammation (IL-6 and TNF-α), and levels of cortisol, epinephrine and norepinephrine. Heart rate (HR), blood pressure (BP), and heart rate variability (HRV) will be assessed as indices of autonomic activation before, during and after the stressor. The speech task is a widely used, highly effective way to investigate stress responses in a laboratory setting. It induces significant increases in cortisol, indices of autonomic activation, and circulating IL-6 and TNFα. Our preliminary data provides novel evidence that this stressor also results in increased cc-mtDNA, which may mediate the increases in circulating levels of IL-6 and TNF-α that peak 40-90 minutes post-stress.

Recruitment: Participants who respond to study advertisements or who are identified by Pitt+Me (a research registry) will be scheduled for a screening telephone call to determine eligibility. After the initial screener by Pitt+Me, eligible volunteers from these sources will be contacted by study staff by text message, telephone, or email. A screening call will be scheduled at which time a member of the research staff will explain the study and administer a telephone screening to assess inclusion/exclusion criteria. If eligible, the staff member will solicit the person's interest in participating. If eligible and interested, male participants will be scheduled for the first laboratory visit and female participants will be asked the predicted date of their next menses so that we may contact them and their laboratory visit can be scheduled during the luteal phase (21-28 days post-menstruation) of their menstrual cycle. Prior to each visit, participants will be asked to abstain from alcohol and vigorous exercise for 24hrs, from non-prescription drugs for two days before testing and from food and caffeine for 3 hrs.

Once the first visit has been scheduled, participants will be mailed/emailed confirmation of the visit, including instructions regarding where to park, public transportation and where to come. This mailing will also include a reminder of the visit preparation instructions (e.g., refrain from alcohol).

Methods: Once the first visit has been scheduled, participants will be mailed/emailed confirmation of the visit, including instructions regarding where to park, public transportation and where to come. This mailing will also include a reminder of the visit preparation instructions (e.g., refrain from alcohol).

Two days before the first laboratory visit, participants will receive a reminder telephone call. On this call, subjects will be screened to determine if they are eligible for the blood draw the following day. Participants who endorse in the past two weeks (1) having an infection, cold or flu, (2) taking antibiotics or glucocorticoids, or (3) having received a vaccination or tattoo will be rescheduled. Participants will also be asked about presence and severity of current symptoms of upper respiratory illness. If they score in a range that is consistent with possible infection, the

visit will be rescheduled. Participants who have taken non-steroidal anti-inflammatories in the past 24 hours will also be rescheduled. If they pass these screenings, standby participants will be confirmed t come in if the primary participant has to be rescheduled.

Participants will attend two laboratory visits at the Behavioral Physiology Laboratory, University of Pittsburgh, scheduled at least one month apart. The first visit will last approximately 4 hours and the second visit approximately 3.5 hours. Both visits will be scheduled in the afternoon starting between 1 and 3 pm to control for diurnal variation in the biological measures of interest. Participants will be asked to avoid eating or drinking anything except water from 12pm on both days of testing. At the first visit, the project coordinator will meet with the volunteer to explain the study and obtain written informed consent according to the University of Pittsburgh Institutional Review Board guidelines.

Following consent, the research nurse will conduct a medical history and medication use screen, and record the participants resting heart rate, blood pressure, height, weight, and body fat. In the event that participants are identified who are ineligible for the study (e.g., due to exclusionary medical or psychiatric conditions or taking excluded medications, with a resting blood pressure >140/90, weight < 110 lbs or BMI equal to or greater than 30), they will be excused from the study at this point.

Next, the blood eligibility assessment will be repeated to confirm that the participant adhered to pre-visit instructions (e.g., no non-prescription medications in the prior 2 days) and did not present with new symptoms of upper respiratory infection. In the event that they are no longer eligible for the blood draw that day, the visit will be rescheduled. Participants who are eligible for the blood draw will next complete demographic questionnaires on the computer. It is estimated that these questionnaires will take 30 minutes to complete.

At both visits, participants will be situated upright in a comfortable chair, where an intravenous catheter will be inserted into the antecubital fossa of one arm for collection of blood samples. Next, a blood pressure cuff (Dinamap) will be placed on the opposite arm from the catheter. Research staff will then apply 11 skin surface electrodes to permit the assessment of heart rate variability [HRV]. Electrodes will be applied to the skin (on the back of the neck, upper chest, shoulders, ribs, and calf area below the knee). The electrodes are the same as those used to record a person's EKG. HRV will be derived from the EKG signal. Following instrumentation, participants will sit quietly for a 30-minute baseline (habituation) period before completing either the speech task or a rest period. Following this, participants will watch a wildlife documentary and complete questionnaires for a 120-min recovery period. Blood samples will be collected at mins. -5 (end of baseline/habituation), and +5, 10, 20, 30, 45, 60, 75, 90, and 120 post the task period. Blood pressure will be recorded twice prior to each of the 10 blood draws. Participants will also complete the Brief Profile of mood State questionnaire on 4 occasions, immediately pre- and post-task, and at 60- and 120-minutes post task. This 39-item adjective checklist provides a measure of emotional state. These time points were chosen to capture both acute and delayed neuroendocrine and inflammatory changes, as well as recovery patterns following standard schedules in the literature.

At one visit, participants will be exposed to the speech task. This is a widely used, highly effective way to investigate stress responses in a laboratory setting. It induces significant increases in cortisol, indices of autonomic activation, and circulating levels of inflammatory mediators. Our preliminary data provides novel evidence that this stressor also results in increased cc-mtDNA. Participants will be told that they will give a speech, recorded by a video camera and in front of an audience (two research staff) trained to assess non-verbal behaviors.

Participants will be given 2 minutes to prepare for the speech defending themselves against an alleged transgression and 3 minutes to deliver it. At the end of the recovery period following the task, all participants will be thoroughly debriefed.

At the other visit, participants will rest quietly for the same period of time with identical assessment in the absence of the stressor. This control session will allow us to assess and control for any possible effects of the passage of time (e.g., circadian variation in cortisol levels), simply being assessed (e.g., discomfort), and the intravenous catheter. Conditions will be counterbalanced in randomized starting order across subjects.

Following completion of the second study visit, participants will be thanked for taking part and receive their payment.

Eligibility Criteria

Inclusion Criteria: Men and premenopausal women between 20 and 50 years of age (equally distributed across decades of age - e.g., 10 men and 10 women aged 20-30) who meet the following criteria:

1. Generally healthy
2. Non-smokers/illicit drug users
3. Blood pressure below 140/90
4. Weight > 110 lbs
5. BMI < 30
6. Fluent in English
7. Women -- regular menstrual cycles over the past 12 months (defined as 21-35 days in length)
8. Able and willing to give informed consent
9. Willing to abstain from alcohol and vigorous exercise for 24 hours, from food and drinks (other than water) for 3 hours and from non-prescription medications (other than oral contraception) for 2 days before testing.
10. Willing to attend two laboratory stress testing sessions, give blood though an intravenous catheter, undergo medical evaluation and complete psychosocial questionnaires.

Exclusion Criteria:
1. Reported history of chronic systemic immune, metabolic or mitochondrial diseases, or chronic diseases that influence the central nervous, autonomic nervous or neuroendocrine systems, e.g., autoimmune disease, chronic infections, cardiovascular disease, diabetes, chronic kidney or liver disease, cancer treatment.
2. Reported psychiatric history of schizophrenia or other psychotic illness, or mood disorder.
3. Resting blood pressure > 140/90 mmHg at baseline testing.
4. Weight < 110 lbs
5. BMI equal to or greater than 30
6. Report currently taking glucocorticoid, anti-inflammatory, anti-retroviral, immunosuppressant, insulin, antiarrhythmic, antihypertensive, oral hypoglycemic, antidepressant, benzodiazepine or prescription weight loss medications or other medications known to influence the immune, autonomic or neuroendocrine systems.
7. For women - Post-menopausal or irregular menstrual cycles over the past 12 months. Report current pregnancy or lactation.

8. Current smokers (defined as having smoked a cigarette in the previous 3 months).
9. Current illicit drug use (defined as reported use of illicit drugs such as marijuana, cocaine or heroin in the previous 3 months).
10. Not fluent in English (have used English in everyday speaking and reading for at least 10 years)
11. Unable or unwilling to give informed consent
12. Unwilling to abstain from alcohol and vigorous exercise for 24 hours, from food and drinks (other than water) for 3 hours and from non-prescription medications (other than oral contraception) for 2 days prior to testing.

Statistical Considerations

For the primary analysis in Aim 1, because we are utilizing a "matched" design (in which each subject serves as his/her own control), we base our power calculations on a simple paired t-test since power formulas are not available for complex models such as the ones we propose to apply to our data. We find that with the proposed sample size n = 60, we have 80% power to detect a normalized difference (in terms of Cohen's d) as small as 0.38. Combined effects sizes from a meta-analysis of the literature [1] yield normalized effect sizes for IL-6 and TNF-α at 0.35 and 0.28, respectively. For ccf-mtDNA, our pilot data give an estimated effect size of 1.13 (see Fig. 4), such that we will be sufficiently powered to detect stress-induced changes, and to detect statistically significant differences in ccf-mtDNA separately in women and in men. In fact, however, we will not have to rely on such a simple analysis, since we are collecting several measurements over time and will be modeling the entire trajectory. With multiple observations, by taking into account necessary covariates and accounting for the correlation structure among repeated observations, we will increase the power of the analysis to detect a change, potentially dramatically, and so we expect to be amply powered. For the secondary analyses, again we will be applying more sophisticated and powerful analyses, but for reference we base our power calculations on Pearson's correlation analysis and find that with the proposed sample size we are powered to detect a "true" correlation coefficient as small as 0.35 (in absolute value), typically considered somewhere between a "small" and "medium" effect size.

Univariate and descriptive analysis will be performed on all dependent variables and, if necessary, normalizing and/or variance stabilizing transformations will be applied to the data before inferential analyses are undertaken. All hypothesis testing will involve two-sided alternative hypotheses with the α level set to 0.05. The analytic strategy for addressing Aim 1 will involve fitting linear mixed models, which allow flexible covariance structures to account for correlation of repeated measures data. Specifically, subject and day (nested within subject) will be the random effects, and condition (stress/control) along with other covariates will be the fixed effects. We will also include time and time^2 (squared) as fixed effects to allow for a nonlinear response. The outcome variables will be the various physiological and psychological responses:

IL-6, TNF-α, ccf-mtDNA, cortisol, epinephrine, norepinephrine HR, BP, momentary negative affect, and HRV.