

Caffeine and stress alter salivary α -amylase activity in young men

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Objective We examined the effects of caffeine and a psychological stressor on salivary α -amylase (sAA) in healthy young males (age 18–30 years) who consumed caffeine on a daily basis.

Methods Using a between-subjects, double-blind, placebo-controlled design, 45 participants received either 200 or 400 mg of caffeine (Vivarin[®]) or placebo, rested for 20 min, and then performed 20 min of mental arithmetic. Saliva samples (assayed for sAA and caffeine), blood pressure, and heart rate were taken before (baseline) and 15 min after the math stressor (stress).

Results Baseline sAA activity did not differ among the treatment groups; however, there was a statistically significant time by caffeine group interaction. Changes in sAA activity across the session were dependent on the amount of caffeine consumed. Following the challenge period, sAA activity among the placebo group was the lowest and sAA activity among the 400 mg treatment group was the highest. Separate repeated-measures ANOVAs conducted for each drug treatment group revealed that sAA activity increased in response to stress and caffeine (*i.e.*, 200 and 400 mg groups) but not to stress alone (*i.e.*, placebo group).

Conclusions Findings provide evidence for acute sAA changes in response to caffeine and stress in habitual caffeine users. Copyright © 2010 John Wiley & Sons, Ltd.

KEY WORDS — blood pressure; caffeine; heart rate; salivary alpha-amylase; stress

INTRODUCTION

Laboratory-based investigations of caffeine are useful in illuminating mechanisms through which caffeine, a methylxanthine, impacts health (for review, see Rodrigues and Klein, 2006). Caffeine is a sympathomimetic that stimulates the central nervous system to release catecholamines and glucocorticoids, elevates blood pressure and increases basal metabolic rate (for reviews, see James, 1997; Mort and Kruse, 2008; Rixsen *et al.*, 2009). Thus, in many ways, caffeine acts as a pharmacologic “stressor” to exact wear and tear on the body. Salivary α -amylase (sAA) is a protein responsible for enzymatic digestion of carbohydrates (Baum, 1993) and mucosal immunity in the mouth (Scannapieco *et al.*, 1993). Recent attention in the neuroendocrine-stress field has been given to sAA because it may be a surrogate marker of sympathetic nervous system (SNS) activity and the ease with which it can be collected and measured in saliva (for reviews see Granger *et al.*, 2007; Nater and Rohleder, 2009; Rohleder and Nater, 2009).

Traditional measures of SNS activity (*e.g.*, plasma catecholamines, sympathetic nerve activity) are themselves invasive and potentially stressful. As a result, scientists have been working to uncover a surrogate marker of SNS activity that can be measured in saliva. A rapidly growing literature suggests that sAA may serve as this surrogate marker (Granger *et al.*, 2007; Nater and Rohleder, 2009; Rohleder and Nater, 2009). The rationale is that catecholamine release from nerve endings in response to SNS activation stimulates salivary gland receptors that, in turn, alter activity of these glands (Nederfors and Dahlof, 1992). Although the main function of sAA is the enzymatic digestion of carbohydrates (Rohleder and Nater, 2009), sAA may be a viable, non-invasive biomarker of stress, as it parallels stress-related increases in plasma norepinephrine about 5–10 min after norepinephrine release (Rohleder *et al.*, 2004). Recently, Nater and Rohleder (2009) suggest that additional pharmacologic studies are needed to provide a clearer picture of sAA activity in response to stress.

With regard to caffeine and sAA, we only could find three published reports, two of which suggest that caffeine stimulates sAA activity (Bishop *et al.*, 2006; Morrison *et al.*, 2003). Bishop *et al.* (2006) reported sAA increases to caffeine administered under prolonged

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exercise in male endurance athletes, which could be considered a stressor. Further, Morrison *et al.* (2003) found that caffeine intake, but not self-reported stress levels, predicted sAA levels among nurses on a pediatric intensive care unit. The stimulating effects of caffeine on sAA may result from caffeine's sympathomimetic effects (Laurent *et al.*, 2000; for review, see Rohleder and Nater, 2009). However, Nater *et al.* (2007) did not find an effect of self-reported caffeine intake on momentary sAA activity in their examination of the diurnal course of sAA. Thus, the limited literature is inconclusive regarding the effects of caffeine on sAA activity. As noted by Rohleder and Nater (2009), it is not known if acute sAA responses to caffeine differ in habitual caffeine users. In addition, the combined effects of caffeine and stress on sAA within a controlled laboratory setting are unknown. Therefore, the present study examined the concurrent effects of caffeine administration and stress on sAA in healthy young men who consumed caffeine on a daily basis (*i.e.*, at least 50 mg of caffeine).

Based on two previous reports that caffeine intake is associated with increases in sAA levels under stress (Bishop *et al.*, 2006; Morrison *et al.*, 2003), we expected caffeine administration in the presence of stress to induce a dose-dependent increase in sAA activity in comparison to placebo. We also expected blood pressure and heart rate (HR) to rise in response to stress and caffeine, a finding consistent with published data (al'Absi *et al.*, 1997; al'Absi *et al.*, 1998; al'Absi *et al.*, 2003; Hartley *et al.*, 2004; Lovallo *et al.*, 2006), and that these elevations would be positively associated with elevated salivary caffeine levels (Laurent *et al.*, 2000).

METHOD

Participants

Forty-five healthy men, 18–30 years of age (mean age 21.16 ± 0.35 years), were recruited to participate in a study examining caffeine and task performance. To minimize the impact of caffeine metabolism and absorption on findings, men only were included in this initial study because of known sex differences in caffeine pharmacokinetics (Abernethy and Todd, 1985). Potential participants were recruited through flyers posted in the local community and on the Penn State campus. Eligibility was determined by a trained research assistant who reviewed the health history of potential participants in a telephone interview. All eligible participants were daily caffeine users who consumed at least 50 mg of caffeine per day (*e.g.*, 12 oz

can caffeinated soda). In addition to daily caffeine use, respondents were asked questions to document significant health problems and the use of medications or drugs that could affect interpretation of neuroendocrine or cardiovascular data, could alter caffeine metabolism, or could harm the participant if caffeine were administered, including: a history of smoking or nicotine use, angina, arrhythmia, medications for blood pressure, diagnosed insulin-dependent diabetes, beta-blocker medication use, inhaled beta agonist use, learning disability, attentional disorder, recent head trauma, history of depression or other psychiatric illness (*e.g.*, anxiety), stroke, seizures, or other focal brain lesion, or a history of other neurological disorders. Likewise, anyone taking the following medications was excluded from participating: oral or parenteral (injected) corticosteroids within 3 months, psychostimulants, over-the-counter stimulants, cold or flu medications, ephedrine or caffeine-containing supplements, cimetidine, quinolones, verapamil, or benzodiazepines. Further, potential participants using psychotropic medications within the previous 8 weeks or with psychiatric hospitalization within the past year were excluded, as were individuals with severe obesity (greater than 140% of ideal body weight). This body weight exclusion was determined by body mass index (BMI; $\text{weight}/\text{height}^2$) as given by the individual over the telephone and then confirmed during the laboratory visit. Individuals also were screened and excluded for symptoms of depression using the Center for Epidemiological Studies Depression Scale (CES-D; Radloff, 1977).

Mean BMI did not differ across experimental groups (see Table 1). Seventy-five per cent of the participants were Caucasian ($N = 34$), 4% were African American ($N = 2$), 16% were Asian ($N = 7$), and 4% were Hispanic ($N = 2$). Ethnicity was equally represented across the three caffeine treatment groups [$\chi^2(6, 45) = 3.11$, n.s.]. All participants were high school graduates; 93% had some college education and 7% of the participants had more than a college education.

Experimental protocol

Eligible participants arrived at the Penn State University General Clinical Research Center (GCRC) at 1300 h and were met by a trained research assistant (CAW) who first obtained informed consent. Participants refrained from taking daily vitamins on the day of their session, ate a low-fat lunch by 1100 h, and avoided caffeine consumption 4 h prior to their laboratory session. Next, participants briefly were interviewed by a certified nurse practitioner to confirm

Table 1. Age and BMI of men in each caffeine treatment group (means \pm SEM)

	Caffeine treatment groups		
	Placebo ($N = 15$)	200 mg ($N = 15$)	400 mg ($N = 15$)
Age (years)	22.69 \pm 0.73	23.91 \pm 0.74	22.36 \pm 0.81
BMI (kg/m^2)	21.33 \pm 0.78	21.00 \pm 0.29	21.13 \pm 0.70

health status and study eligibility. Participants then were asked to complete a demographic survey and a comprehensive measure of daily caffeine use. Following completion of the questionnaires, a standard blood pressure cuff (Dinamap Compact Blood Pressure Monitor, Critikon, Tampa, FL) was placed on the non-dominant arm to collect systolic blood pressure (SBP), diastolic blood pressure (DBP), and HR. This automated oscillometric monitor has been shown to yield blood pressure values that are highly correlated with intra-arterial and ambulatory blood pressure measurements (Borow and Newburger, 1982; Mueller *et al.*, 1997). After cuff placement, a sample blood pressure reading was taken to ensure that blood pressure levels fell within an acceptable range (*i.e.*, SBP < 140 mmHg, DBP < 90 mmHg, HR < 100 beats per minute). Participants whose cardiovascular readings did not meet these criteria were excluded from the study ($N = 3$); their data are not reported here.

Baseline. Participants were asked to sit quietly for 10 min while five baseline blood pressure readings were taken automatically at 2-min intervals. Next, participants were asked to give a saliva sample by rolling a cotton swab across their tongue (without chewing on the swab) for 2 min and then placing it into a saliva collection tube (Salivette; Sarstedt, Inc., Newton, NC; for review of sAA saliva collection methods see Rohleder and Nater, 2009). Saliva samples immediately were placed on ice until transferred to a -80°C low-temperature freezer for later assessment of sAA and caffeine. Participants then were asked to complete a computerized working memory task, reaction time, and signal detection task (results not reported here), during which time blood pressure and HR were recorded every 2 min. These tasks took no more than 15 min to complete.

Caffeine administration. Following the computer tasks, participants were asked to swallow two gelatin capsules with a glass of water. The capsules were green in color, made of vegetable oil, and easily digestible (Size 1 K-Caps Vegetarian Capsules, Capsuline[®], Pompano Beach, FL). Each capsule contained either methylcellulose (placebo; Spectrum Chemicals, Gardena, CA) or a

200 mg Vivarin[®] (GlaxoSmithKline, Philadelphia, PA) pill. Using a randomized double-blind procedure, participants in the placebo group ($N = 15$) received two methylcellulose capsules, participants in the 200 mg caffeine group ($N = 15$) received one methylcellulose and one caffeine capsule, and participants in the 400 mg caffeine group ($N = 15$) received two caffeine capsules. This caffeine administration paradigm was selected based on previously published studies (*e.g.*, Lane *et al.*, 2002; Lieberman *et al.*, 2002) and to more closely parallel caffeine consumption outside the laboratory where individuals consume caffeine in the form of beverages (*e.g.*, sodas, coffee) and food (*e.g.*, chocolate).

After capsule administration, participants were asked to rest for 20 min to allow for adequate caffeine absorption and to ensure that participants were completing the mental arithmetic task when plasma caffeine levels were on the ascending limb of the absorption curve (Bonati *et al.*, 1982; Liguori *et al.*, 1997); blood pressure and HR readings were taken every 2 min.

Math challenge. Next, a trained investigator (LCK) entered the room to administer the stressor which was a serial subtraction task drawn from the Trier Social Stress Task (Kirschbaum *et al.*, 1993). Specifically, participants were asked to count backwards by 7's and 13's two different times; each counting segment lasted for 4 min. Task performance was voice recorded on a digital camera and laptop computer for later assessment of accuracy and speed (for task performance results see Bennett *et al.*, 2006). This challenge session took 25 min to complete; blood pressure and HR were recorded every minute during this time period.

Recovery. Following completion of the math challenge, participants again were asked to complete the computer cognitive tasks and then the second saliva sample was collected. Next, participants were asked to rest for a 15-min recovery period. Blood pressure and HR were recorded every 2 min throughout the computer and recovery periods. Participants were paid \$50 for their time and were informed of their caffeine condition by a nurse at the GCRC. All procedures were

reviewed and approved by The Pennsylvania State University Institutional Review Board.

Physiological measures

Salivary caffeine. Salivary caffeine levels were determined using liquid chromatography (LC) coupled with tandem mass spectrometry (LC-MS/MS) developed in the Clinical Pharmacology Research Laboratories at the University of California, San Francisco. Stable isotope-labeled analogs of caffeine were used as internal standards. Following protein precipitation, the salivary samples (0.2 mL) were treated with phosphate buffer and caffeine extracted with a mixture of methylene chloride, ethyl acetate, and isopropyl alcohol. The extracts were evaporated, reconstituted in the LC mobile phase, and injected into the LC-MS/MS system. The mass spectrometer was operated using atmospheric pressure chemical ionization, and selected reaction monitoring was used for quantization. Calibration curves were constructed using the peak area ratio of analyte/internal standard and linear regression. Limits of quantization for the analytes in saliva are 10 ng/mL. Precision (within-run, percent coefficient of variation, $n=6$) ranged from 1.7 to 10.3%, and accuracy (percent of expected value) ranged from 88 to 118% for concentrations from 10 to 5000 ng/mL.

Salivary α -amylase (sAA). sAA was measured using a kinetic reaction assay that employs a chromogenic substrate, 2-chloro-*p*-nitrophenol, linked to maltotriose (Granger *et al.*, 2006). The enzymatic action of sAA on this substrate yields 2-chloro-*p*-nitrophenol, which can be spectrophotometrically measured at 405 nm using a standard laboratory plate reader. The amount of sAA activity present in the sample is directly proportional to the increase (over a 2 min period) in absorbance at 405 nm. Results are computed in U/mL of sAA using the formula: [Absorbance difference per minute \times total assay volume (328 mL) \times dilution factor (200)]/[millimolar absorptivity of 2-chloro-*p*-nitrophenol (12.9) \times sample volume (0.008 mL) \times light path (0.97)]. Intra-assay variation (CV) computed for the mean of 30 replicate tests was less than 7.5%. Inter-assay variation computed for the mean of average duplicates for 16 separate runs was less than 6%.

Blood pressure and heart rate. Aggregation across two measures of basal resting SBP and DBP in a laboratory setting has been shown to provide within-subject reliability of +0.90 or better (Llabre *et al.*, 1988). Therefore, SBP and DBP readings, along with

HR, were averaged across each experimental time period to derive mean baseline (5 readings), challenge (16 readings), and recovery (6 readings) measures for each participant.

Statistical analyses

Caffeine dosage (mg/kg) for each participant was determined by dividing the amount of caffeine (mg) by the participant's body weight (kg) to ensure consistency in caffeine administration across caffeine treatment groups. Consequently, one participant in the 400 mg group with a BMI > 31 received a much lower dosage (3.55 mg/kg) than did the other men ($N=14$) in this treatment condition (5.90 ± 0.92 mg/kg, range 4.02–6.68 mg/kg). In fact, this participant's individual dosage was consistent with men in the 200 mg group ($N=15$, 2.67 ± 0.11 mg/kg, range 2.11–3.61 mg/kg). Therefore, we moved this participant into the low caffeine dose group (*i.e.*, 200 mg) for data analyses. It is important to note, however, that the following results did not change as a result of moving this participant into the low dose caffeine group.

Square root transformations were applied to the sAA data because they were not normally distributed (Gordis *et al.*, 2006; Granger *et al.*, 2007); this transformation resulted in a normal distribution of the data. Thus, all sAA statistical analyses are based on square root-transformed values; raw sAA values are reported unless otherwise noted.

Separate repeated-measures analysis of variance (ANOVA), with Caffeine Treatment (three levels) as the independent measure and Time as the within-subject variable, were conducted to examine group differences in SBP, DBP, HR, sAA, and caffeine levels during the baseline, challenge (SBP, DBP, HR only), and recovery phases of the experiment. When appropriate, statistical interactions were examined using separate one-way ANOVAs, Tukey's honestly significant difference (HSD), and Bonferroni *post-hoc* analyses. All significance tests were two-tailed and evaluated at $\alpha = 0.05$.

RESULTS

Manipulation check

Caffeine administration. Saliva samples taken at baseline (*i.e.*, before pill administration) and after the challenge period confirmed caffeine administration (see Table 2). Specifically, repeated-measures ANOVA revealed a significant effect of time [$F(1,42) = 218.98$, $p < 0.05$] and a time by caffeine group interaction

Table 2. Laboratory-administered caffeine dosage (mg/kg), baseline and recovery salivary caffeine (ng/mL) levels and baseline, stress, and recovery systolic (SBP) and diastolic (DBP) blood pressure (mmHg) and HR (beats per minute; BPM) levels among men in each caffeine treatment group (means \pm SEM)

	Caffeine treatment groups		
	Placebo (N = 15)	200 mg (N = 16)	400 mg (N = 14)
Caffeine Dosage (mg/kg)	0.00 \pm 0.00	2.72 \pm 0.11	5.90 \pm 0.20 ^a
Salivary Caffeine (ng/mL)			
Baseline	271.78 \pm 78.00	256.31 \pm 67.83	338.76 \pm 112.06
Recovery	195.27 \pm 60.95	3547.70 \pm 207.00	6751.16 \pm 699.10 ^b
Systolic Blood Pressure (mmHg)			
Baseline	115.92 \pm 2.56	114.95 \pm 2.84	118.25 \pm 2.22
Stress	133.79 \pm 3.90	132.27 \pm 4.04	133.82 \pm 3.59
Recovery	119.18 \pm 3.06 ^c	126.60 \pm 3.28 ^{c,d}	123.83 \pm 2.33 ^{c,d}
Diastolic Blood Pressure (mmHg)			
Baseline	68.60 \pm 1.70	67.46 \pm 1.58	68.00 \pm 1.76
Stress	79.24 \pm 2.08	77.44 \pm 1.92	75.45 \pm 2.07
Recovery	71.88 \pm 1.78 ^c	74.04 \pm 1.98 ^c	70.61 \pm 1.69 ^c
HR (BPM)			
Baseline	64.15 \pm 2.60	68.91 \pm 3.05	68.66 \pm 2.52
Stress	74.57 \pm 2.58	72.44 \pm 2.32	76.99 \pm 2.72
Recovery	64.88 \pm 2.29 ^c	64.45 \pm 2.19 ^c	68.68 \pm 2.45 ^c

^aCaffeine treatment effect (200 mg < 400 mg), $p < 0.001$.

^bCaffeine treatment effect (0 mg < 200 mg < 400 mg), $p < 0.001$.

^cTime effect, $p < 0.05$.

^dBaseline < Recovery, $p < 0.05$.

[$F(2,42) = 72.39$, $p < 0.05$] such that, as expected, caffeine levels increased across the session in a dose-dependent manner.

At the end of the session, a nurse asked participants to guess whether they received placebo, 200 mg caffeine or 400 mg caffeine pills. Twenty (44.4%) participants correctly guessed which pills they had received; this number was equally distributed across the three drug conditions [$\chi^2(2,45) = 2.11$, n.s.].

Blood pressure and heart rate. As expected, SBP, DBP, and HR changed significantly across baseline, stress, and recovery [F 's(2,84) > 38.14, p 's < 0.05, respectively] (see Table 2). Caffeine administration altered SBP changes over the laboratory session [$F(4,84) = 2.61$, $p < 0.05$] such that SBP levels during recovery did not return to baseline among participants administered 200 and 400 mg of caffeine [F 's(2,26) > 26.05, p 's < 0.05]. Caffeine did not interact with time with regard to DBP or HR.

Main findings

Salivary α -amylase (sAA). Baseline sAA activity did not differ among the caffeine groups. Overall, the math challenge increased sAA activity across all participants [$F(1,42) = 437.25$, $p < 0.05$]. In addition, there was a statistically significant time by caffeine group interaction [$F(1,42) = 132.43$, $p < 0.05$] where sAA activity

increased across the session in a dose-dependent manner (see Figure 1). Specifically, following the challenge period, sAA activity was significantly different across the three treatment groups, with sAA activity among the placebo group being the lowest and sAA activity among the 400 mg treatment group being the highest. Separate repeated-measures ANOVAs were conducted for each drug treatment group to further explore this time by caffeine interaction. sAA activity increased in response to stress and caffeine but not to stress alone [200 mg:

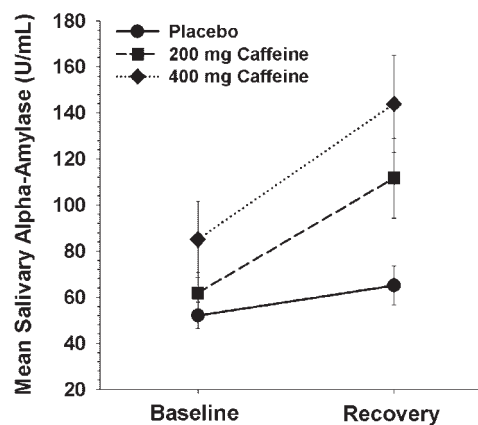


Figure 1. sAA (U/mL) among men administered no caffeine, 200 or 400 mg of caffeine at baseline (before caffeine administration) and recovery (15 min following cessation of the stressor) (means \pm SEM)

$F(1,15) = 587.06, p < 0.05$; 400 mg: $F(1,13) = 171.84, p < 0.05$; placebo: $F(1,14) = 4.28, n.s.$].

Pearson product-moment correlations were conducted to examine the relationship between sAA and caffeine levels. Baseline (*i.e.*, pre-caffeine, pre-stress) caffeine levels were not correlated with baseline sAA activity [$r(45) = +0.07, n.s.$] whereas recovery (*i.e.*, post-caffeine administration, post-stress) caffeine levels were significantly, positively correlated with recovery sAA activity [$r(45) = +0.33, p < 0.05$] (see Figure 2).

DISCUSSION

We investigated the concurrent effects of caffeine administration and stress on sAA in healthy young men who consumed caffeine on a daily basis. As predicted, caffeine administration in the presence of stress induced a dose-dependent increase in sAA activity in comparison to placebo. These findings extend the two prior reports that caffeine intake with stress exposure is associated with increased sAA levels (Bishop *et al.*, 2006; Morrison *et al.*, 2003) in several ways. These data demonstrate stress and caffeine's effects on sAA in a controlled laboratory setting where (1) caffeine levels were manipulated in a dose-dependent manner, (2) participants were habitual caffeine consumers, and (3) salivary caffeine levels were measured, which can account for individual differences in caffeine pharmacokinetics. Importantly, salivary caffeine levels following drug administration (*i.e.*, post-stress) were positively correlated with sAA activity. In contrast, baseline caffeine levels (*i.e.*,

caffeine levels in habitual caffeine users *prior to* laboratory-administered caffeine), were not associated with baseline, pre-stress sAA activity. Similar to a recent report by Stroud *et al.* (2009) with children and adolescents, sAA activity did not change in response to the mental arithmetic challenge (*i.e.*, "performance stressor") among participants in the placebo condition. Unfortunately, our experimental paradigm makes it difficult to conclude whether caffeine alone or in conjunction with stress is responsible for increased sAA activity from baseline to post-stress. Additional studies that include non-stress conditions are needed to better understand the unique contributions of caffeine to sAA activity. For example, stress exposure can increase caffeine metabolism rates, which can alter caffeine bioavailability in unexpected ways. Thus, it is possible that caffeine intake in the presence of stress, but not alone, may alter sAA activity.

The present study addressed the hypothesis that acute caffeine administration in habitual caffeine consumers plus acute stress exposure elevates sAA activity. Unlike our findings and that of others (Bishop *et al.*, 2006; Morrison *et al.*, 2003), Nater *et al.* (2007) did not find a relationship between daily caffeine intake and diurnal sAA levels. There are important differences between Nater *et al.*'s (2007) field study and our laboratory-based experiment. First, self-reported caffeine levels in Nater *et al.* (2007) most likely were lower than the laboratory-administered caffeine levels in our study, particularly within the time frame of caffeine administration. In other words, 200 mg caffeine ingestion in a pill is comparable to drinking an 8 oz cup of coffee in less than ~15 s, which will yield peak caffeine levels that are higher than sipping a cup of coffee over a 30- to 60-min window. Thus, it is possible that there is a threshold level of caffeine administration needed before sAA levels are altered. Second, caffeine pharmacokinetics may have affected Nater *et al.*'s (2007) ability to detect an effect of caffeine on sAA. We designed our sAA assessment and onset of stress exposure around the time course of caffeine absorption and distribution to maximize the physiologic effects of a single, large dose of caffeine. We also controlled the time of day of caffeine and stress exposure, and specifically measured salivary caffeine levels. In contrast, Nater *et al.* (2007) obtained sAA levels and self-reported caffeine intake amounts across the day, which could not account for individual differences in the duration of caffeinated beverage consumption in connection with each sAA assessment or the concurrent experience of stress during caffeine intake. Third, our participants abstained from caffeine for 4 h prior to caffeine consumption in the laboratory

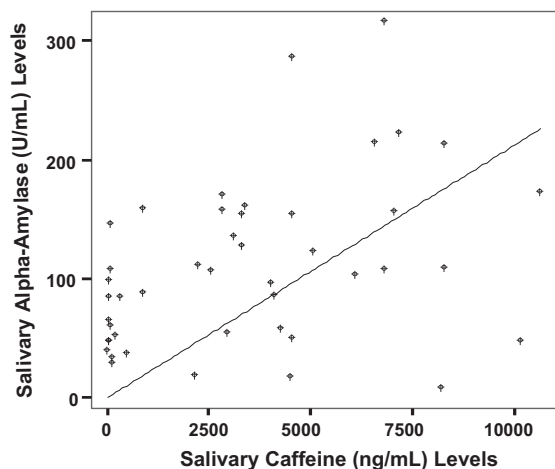


Figure 2. Relationship between sAA (U/mL) and caffeine (ng/mL) levels during recovery ($N = 45$)

whereas participants in Nater *et al.* (2007) freely drank caffeine across the day. It is possible that sAA responses to acute caffeine administration habituate across the day, a hypothesis that needs further investigation. Finally, unlike Nater *et al.* (2007) who included both men and women in their study population, our study only examined sAA responses to stress and caffeine administration among men. There are known sex differences in caffeine absorption, distribution, metabolism, and elimination rates (Abernethy and Todd, 1985) that could mediate or moderate the effects of caffeine on sAA levels. Taken together, it seems premature to conclude that caffeine alone does not alter sAA levels. Additional laboratory-based studies that include sAA assessments in response to multiple dosages of caffeine administered across various time points throughout the day, as well as salivary caffeine level assessment, are needed in both men and women before a definite conclusion can be made regarding the independent relationship between sAA levels and caffeine consumption.

As mentioned earlier, the stimulating effects of stress and caffeine on sAA may be the result of SNS activation (Laurent *et al.*, 2000; for review, see Rohleder and Nater, 2009). sAA activity can be triggered through SNS activation, though the parasympathetic nervous system (PNS) potentially could affect sAA activity independently (*e.g.*, Ekström *et al.*, 1996; Jensen *et al.* 1991). Caffeine is a known sympathomimetic, thus it is very likely that combined SNS stimulation by caffeine and stress led to the observed increase in sAA activity in this study. Our study only included sAA, blood pressure, and HR assessments of autonomic nervous system (ANS) activity (*i.e.*, SNS and PNS) activity. Laboratory-controlled studies are needed that include multiple ANS biomarkers (*e.g.*, plasma epinephrine, plasma norepinephrine, skin conductance, cardiac pre-injection period, HR variability) in the presence of caffeine, both with and without stress, to better understand the physiological mechanisms through which caffeine (with and without stress) alters sAA and ANS activity. The additional advantage of including these multiple biomarkers is that a better understanding of the unique contributions of the SNS and PNS to sAA activity might be developed.

To our knowledge, this is the first report on the effects of caffeine administration and stress on sAA in healthy young men who consume caffeine on a daily basis. The physiological (*e.g.*, blood pressure, HR) effects of caffeine can be attenuated in habitual caffeine users. Thus, our participants were asked to refrain from caffeine intake for 4 h prior to their

laboratory session to minimize any habituation effects of caffeine intake. Further, we limited the range of self-reported daily caffeine intake (mg/day) in an attempt to minimize individual variance in physiological reactivity to the laboratory administered caffeine. Specifically, participants were screened by telephone to ensure that they consumed self-reported 50 mg of caffeine per day and no more than 700 mg/day; eligible participants went on to complete a detailed daily caffeine intake during their laboratory session. Data from this detailed questionnaire revealed that self-reported daily caffeine intake actually ranged from a cup of tea (40 mg) to just over 700 mg/day. One participant reported more than 1000 mg of caffeine/day but we suspect he misinterpreted the instructions on how to complete the caffeine intake inventory. Regardless, to determine whether self-reported daily caffeine intake was consistent across the three drug treatment groups, we created a median split on self-reported daily caffeine intake and conducted a Chi-square test to ensure that high and low daily caffeine consumers were equally distributed across the three drug treatment groups, which they were [$\chi^2(2,45) = 2.19$, n.s.]. We also conducted separate one-way ANOVAs with high/low daily caffeine intake group as the independent variable and baseline (*i.e.*, pre-caffeine treatment) sAA levels and recovery (*i.e.*, post-stress, post-caffeine administration) sAA levels as the dependent measures to examine the influence of self-reported daily caffeine intake on sAA levels. Both analyses were not statistically significant. A repeated-measures ANOVA with the same independent variable also revealed that there was no high/low daily caffeine intake interaction with time on sAA levels. Taken together, these results suggest that self-reported daily caffeine intake levels did not alter the sAA results in this study.

A particular strength of this study is that salivary caffeine levels prior to laboratory caffeine administration were measured. Thus, these basal caffeine levels are a marker of daily caffeine intake on the day of the study. Importantly, basal salivary caffeine levels were not correlated with basal sAA levels which gives us further confidence that daily caffeine intake did not affect the current results. Although expensive, inclusion of salivary caffeine levels (and their metabolites) in sAA studies would be invaluable in determining the relationship between salivary sAA activity and caffeine intake.

Chronic stress experiences also can alter momentary sAA activity compared to individuals with low chronic stress levels (Nater *et al.*, 2007). Unfortunately, we did not evaluate chronic stress levels in participants when

they arrived in the laboratory. Although baseline sAA activity did not differ across drug treatment groups prior to stress and caffeine/placebo administration, it remains possible that individual differences in chronic stress levels influenced sAA levels following caffeine and stress administration. Future studies should include multilevel assessments of stress both inside and outside the laboratory to help delineate the independent effects of stress and caffeine on sAA levels.

The present results are intriguing and present more questions than answers with regard to the relationship among caffeine intake, stress and sAA activity. Nonetheless, these findings suggest that sAA be considered as a valuable biomarker in biobehavioral studies on the health effects of caffeine and that a multilevel biomarker and psychosocial assessment approach that includes men and women, both in and out of the laboratory, will be invaluable in moving this field forward.

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