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Acute stress responses in salivary alpha-amylase predict increases of plasma norepinephrine

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ABSTRACT

Current biobehavioral research increasingly employs salivary alpha-amylase (sAA) as a surrogate marker for sympathetic nervous system (SNS) activity. While different lines of evidence point to the validity of this assumption, the literature is inconsistent with regard to associations of sAA with well-established SNS indicators, such as plasma norepinephrine (NE) or epinephrine (E). Small samples as well as application of different stress paradigms might be responsible. This study therefore set out to examine the relation between stress-induced sAA activity with NE and E by exposing a larger and less constrained sample to an effective stress protocol. Sixty-six healthy participants (mean age 24.30 ± 4.24 yrs), including $n = 40$ women, $n = 26$ men, $n = 18$ oral contraceptive (OC) users, and $n = 15$ habitual smokers, were recruited and subjected to the Trier Social Stress Test (TSST). Saliva and blood samples were taken at four time points throughout the experiment for later analysis of sAA activity and NE/E concentration, respectively. As expected, sAA, NE, and E showed significant increases in response to the acute stress induction (all $p < 0.001$). Regression analyses (controlling for age, BMI, sex, smoking and OC) revealed that stress responses in sAA significantly predicted stress responses in NE ($r = 0.326$; $p = 0.025$). Interestingly, stress responses in E predicted NE to a lesser extend ($\beta = 0.265$; $p = 0.064$). E responses showed no association with sAA ($\beta = 0.265$; $p = 0.064$). Higher sAA levels were found in habitual smokers ($F = 4.27$; $p = 0.043$) and in individuals with lower BMI ($F = 2.81$; $p = 0.099$). In conclusion, current data clearly show an association between stress responses of sAA and plasma NE. This relationship is stronger than the association of norepinephrine and epinephrine responses, thus placing the predictive power of sAA well within the expected range for different SNS markers.

1. Introduction

An exponentially growing body of literature discusses the use of salivary alpha-amylase (sAA) to serve as an alternative biochemical indicator of sympathetic nervous system (SNS) activity (see reviews in Nater and Rohleder, 2009; Rohleder and Nater, 2009). Yet, some questions remain unanswered, such as the relationship between salivary flow rate and parasympathetic activation, and other methodological aspects (see comments by Bosch et al., 2011). In particular, it has been noted that if sAA was a legitimate index for SNS activity, there should be consistent significant correlations between stress-induced sAA concentrations and SNS activity reflected for example in plasma concentrations of the catecholamines norepinephrine (NE), or epinephrine (E), or by other peripheral SNS activity markers. The limited available literature is currently inconclusive regarding this relationship.

Salivary alpha-amylase is a major enzyme in saliva secreted from the secretory granules (Zakowski and Bruns, 1985). The interest in sAA as an alternative stress marker has evolved from the observation that sAA and catecholamines share some common characteristics regarding baseline activity and stress-induced response patterns (e.g. Balodis et al., 2011; Bosch et al., 1996; Chatterton, 1996, 1997; Gordis et al., 2006; Harmon et al., 2008; Kivlighan and Granger, 2006; Nater et al., 2005, 2006, 2007; Rohleder et al., 2004, 2006a,b; Steerenberg et al., 1997; Stroud et al., 2006; van Stegeren et al., 2006). Additionally, pharmacological challenge tasks revealed that sAA levels increase in response to the application of beta-adrenergic agonists (Gallacher and Petersen, 1983; Speirs et al., 1974), or alpha-2-adrenergic receptor antagonists (Ehlert et al., 2006), or are suppressed by the administration of beta-adrenergic antagonist (Nederfors and Dahlof, 1992; Nederfors et al., 1994; Speirs et al., 1974; van Stegeren et al., 2006). Finally, studies evaluating the association between sAA and other indicators of SNS activity, reported positive correlations of stress induced

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sAA levels with measures of sympatho-vagal balance (Nater et al., 2006), with increases in heart rate (HR), and negative correlations with decreases in heart rate variability (HRV) (Bosch et al., 2003), as well as with basal skin conductance level (El-Sheikh et al., 2008).

Despite the evidence summarized above, data on correlations between stress-induced sAA and plasma catecholamines NE/E reported by previous studies has been inconclusive so far, weakening the confidence in the use of sAA as an SNS marker. Chatterton et al. (1996), who investigated stress-induced increases of sAA with plasma NE/E found significant correlations between these parameters only when stress was induced by a physical stressor (i.e. running exercise), but not when the stress was induced by a psychological stressor (i.e. examination) (1996). However, it may be argued that the applied psychological stressor was too mild to exert strong enough stress responses in both parameters. Indeed, Rohleder et al. (2004), who applied an evaluated standardized stressor (Trier Social Stress Test, TSST), which is known to reliably induce strong physiological stress responses (see Dickerson and Kemeny, 2004), found a significant correlation between stress-induced sAA increases and plasma NE responses to stress in their sample ($N = 12$). However, Nater et al. (2006), who were applying the same stress paradigm in a larger sample ($N = 30$), reported no significant correlations between sAA and NE/E. Yet, given the highly homogenous sample in that study, i.e. all healthy, non-smoking men between 19 and 28 years, generalizability of the results beyond this very definite sample is highly restricted. Finally, Wetherell et al. (2006), who were investigating both, men and women in their study, found no significant correlations between plasma NE and sAA in their healthy sample ($N = 24$), either. Here again, it may be argued that the applied stressor in that study, a single inhalation of 35% CO_2, might not have been comparable to typically used psychosocial stressors.

Based on the fact that only very few studies have tested the expected relationship between sAA and NE/E stress responses, and that these studies were either small and/or used weaker stress paradigms, we set out to test this relationship with a significant stressor in a larger sample. Besides the need to include male *and* female participants (e.g. van Stegeren et al., 2008; Yamaguchi et al., 2006), we decided to also include individuals with known or expected alterations in autonomic functioning in general, or sAA alterations in particular. We assumed that the inclusion of individuals with some autonomic alterations, such as females using hormonal contraceptives (e.g. Blum et al., 1988; Giraldo et al., 2008), or habitual smokers (e.g. Callegari and Lami, 1984; Goi et al., 2007; Granger et al., 2007; Greabu et al., 2007; Nagler et al., 2000; Zappacosta et al., 2002) would increase generalizability of the results. It was therefore the aim of the present study to investigate stress responses of sAA and the plasma catecholamines NE and E to a well-evaluated and highly effective standardized stress paradigm in a larger and more heterogeneous sample of healthy male and female participants, females taking hormonal contraceptives as well as habitual smokers.

2. Methods and materials

2.1. Study participants

Sixty-six healthy participants were recruited for the current study. The sample consisted of 40 women and 26 men. Twenty-two female participants were free of hormonal contraceptives and were menstruating regularly. They were examined in the luteal phase (day 22–27) of their menstrual cycle. All other female participants ($n = 18$) were using oral contraceptives (OC user). Females using OC were included in the study because of evidence showing elevated catecholamine levels in these individuals (Blum et al., 1988; Giraldo et al., 2008). Fifteen participants (6 women, 9 men) were habitual smokers. Habitual smoking was defined as smoking fifteen or more cigarettes a day in the past two years or longer, with a minimum of 1 mg nicotine content per cigarette. None of the female smokers were OC users. Smokers were included in the study, because it has been shown that habitual smokers show both, decreased baseline sAA activity (Callegari and Lami, 1984; Goi et al., 2007; Granger et al., 2007; Weiner et al., 2008), and decreased sAA acute responses (Greabu et al., 2007; Nagler et al., 2000; Zappacosta et al., 2002). Participants were recruited at the University of Düsseldorf and screened for previous or current health issues. Participants were included in the study if they were free of chronic diseases (psychiatric, endocrine, cardiovascular, and others), chronic stress (as tested by the Trier Inventory for Assessment of Chronic Stress, TICS, Schulz and Schlotz, 1999), medication (psychoactive, beta-blockers, glucocorticoids), in a BMI range between 17 and 27 kg/m^2, and an age range between 18 and 42 years. All participants were informed about the nature and procedures of the study orally and in written form. The applied stress paradigm was not described in detail until the start of the stress paradigm. Written informed consent was obtained from all participants. Participants received EUR 50 for their participation in the study. The study was conducted in accordance with the declaration of Helsinki. The study protocol was approved by the Ethics Committee of the German Psychological Association (DGPs).

2.2. Procedures

If basic eligibility requirements (e.g. age, BMI, free of medication) were confirmed in a telephone screening, participants were scheduled for an appointment on a weekday in the laboratory. To minimize potential effects of circadian rhythmicity in the measured biological parameters, all appointments were scheduled at the same time in the afternoon between 15:00 h and 17:00 h. Furthermore, participants were asked to refrain from drinking alcoholic or caffeinated beverages 24 h prior the experiment. Moreover, participants were instructed not to eat or brush their teeth 1 h before coming to the laboratory.

Upon arrival at the laboratory and after providing written informed consent, individuals underwent a brief health examination. If all inclusion criteria were met, participants were instructed in the saliva sampling method, which included placing cotton swabs (Salivettes, Sarstedt, Nümbrecht, Germany) in their mouth and circulating it for 2 min, as recommended by Harmon et al. (2008). A catheter (Vasofix Braunüle, Braun, Melsungen, Germany) was inserted into an antecubital vein. A first blood draw and saliva sample was taken 45 min after catheterization (−1 min). Blood was collected into EDTA coated Monovettes (Sarstedt, Nümbrecht, Germany). Immediately after the blood draw and saliva sample, all participants underwent the psychosocial stress paradigm "Trier Social Stress Test" (TSST). The TSST is a standardized laboratory stress protocol, which consists of a short introduction given by the main experimenter, a preparation period in silence, and a public speech followed by a mental arithmetic task (each of 5 min duration) in front of an audience (Kirschbaum et al., 1993). Participants were informed that the audience, consisting of two persons in white laboratory coats, were experts specifically trained in analyzing verbal- and non-verbal behavior. Participants were additionally informed that their performance would be videotaped. The TSST is considered a reliable instrument and proven to induce strong HPA axis and SNS stress responses (see Dickerson and Kemeny, 2004). Immediately after the TSST, a second blood draw and saliva sample was taken (+1 min). Two additional blood draws and saliva samples were taken +20 and +45 min after cessation of the TSST.

2.3. Biochemical analyses

2.3.1. Salivary alpha-amylase

Saliva samples were immediately stored at −20 °C. For batch analysis at the end of data collection, salivettes were thawed and centrifuged at 2000 × *g* for 5 min. This procedure resulted in a clear supernatant of low viscosity. Salivary alpha-amylase measurement was completed using an enzyme kinetic method as described previously (Bosch et al., 2003; Rohleder and Nater, 2009). Saliva was processed on a Genesis RSP8/150 liquid handling system (Tecan, Crailsheim, Germany). Saliva was diluted at 1:625 with ultrapure water by the liquid handling system. Twenty microliters of diluted saliva and standard were then transferred into standard transparent 96-well microplates (Roth, Karlsruhe, Germany). Standard was prepared from "Calibrator f.a.s." solution (Roche Diagnostics, Mannheim, Germany) with concentrations of 326, 163, 81.5, 40.75, 20.38, 10.19, and 5.01 U/L alpha-amylase, respectively, and ultrapure water as zero standard. Afterwards, 80 μl of substrate reagent (alpha-amylase EPS Sys; Roche Diagnostics, Mannheim, Germany) were pipetted into each well using a multichannel pipette. The microplate containing sample and substrate was then heated to 37 °C by incubation in a waterbath for 90 s. Immediately afterwards, a first interference measurement was obtained at a wavelength of 405 nm using a standard ELISA reader (Anthos, Labtech HT2, Anthos, Krefeld, Germany). The plate was then incubated for another 5 min at 37 °C in the waterbath, before a second measurement at 405 nm was taken. Increases of absorbance in samples were transformed to alpha-amylase concentrations using a linear regression computed against the standard curve on each microplate (Graphpad Prism 4.0c for Mac OS X, Graphpad Software, San Diego, CA). Inter- and intraassay variation was below 10%.

2.3.2. Norepinephrine and epinephrine

EDTA-treated blood samples were placed on ice immediately after the blood draw and immediately centrifuged at 2000 × *g* and 4 °C for 10 min. Plasma was aliquoted and stored at −80 °C until the end of data collection. Plasma samples were analyzed for NE and E by high-pressure liquid chromatography (HPLC; detection limit: 0–25 pg/ml) at the Laboratory for Stress Monitoring (Göttingen, Germany).