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Norepinephrine infusion with and without alpha-adrenergic blockade by phentolamine increases salivary alpha amylase in healthy men

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Abstract
Background: Mental stress reliably induces increases in salivary alpha amylase (sAA), a suggested surrogate marker for sympathetic nervous system (SNS) reactivity. While stress-induced sAA increases correlate with norepinephrine (NE) secretion, a potential mediating role of noradrenergic mechanisms remains unclear. In this study, we investigated for the first time in humans whether a NE-stress-reactivity mimicking NE-infusion with and without alpha-adrenergic blockade by phentolamine would induce changes in sAA.
Methods: In a single-blind placebo-controlled within-subjects design, 21 healthy men (29—66 years) took part in three different experimental trials varying in terms of substance infusion with a 1-min first infusion followed by a 15-min second infusion: saline-infusion (trial-1), NE-infusion (5 μg/min) without alpha-adrenergic blockade (trial-2), and with phentolamine-induced non-selective blockade of alpha1- and alpha2-adrenergic receptors (trial-3). Saliva samples were collected immediately before, during, and several times after substance infusion in addition to blood pressure and heart rate readings.

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Results: Experimental trials significantly differed in sAA reactivity to substance-infusion ($p = .001$) with higher sAA reactivity following NE-infusion with (trial-3; $p = .001$) and without alpha-adrenergic-blockade (trial-2; $p = .004$) as compared to placebo-infusion (trial-1); sAA infusion reactivity did not differ between trial-2 and trial-3 ($p = .29$). Effective phentolamine application was verified by blood pressure and heart rate infusion reactivity. Salivary cortisol was not affected by NE, either with or without alpha-adrenergic-blockade.
Conclusions: We found that NE-infusion stimulates sAA secretion, regardless of co-administered non-selective alpha-adrenergic blockade by phentolamine, suggesting that the mechanism underlying stress-induced sAA increases may involve NE.

1. Introduction

Salivary alpha-amylase (sAA) is a digestive enzyme secreted from salivary glands in oral cavity that has been proposed as a sensitive surrogate marker for activity of the sympathetic nervous system (SNS) during stress (Nater and Rohleder, 2009). Accumulating evidence suggests elevated sAA secretion by salivary glands under both physiological and psychological stress when the SNS is activated (Bosch et al., 1996; Chatterton et al., 1997; Bosch et al., 2003; Rohleder et al., 2004; Nater et al., 2006; van Stegeren et al., 2006; Thoma et al., 2012). Indeed, several studies found associations between sAA and plasma norepinephrine (NE) levels with a similar stress reactivity kinetics (Chatterton et al., 1996; Rohleder et al., 2004; Thoma et al., 2012), although not unequivocally (Nater et al., 2006; Wetherell et al., 2006). Notably, SNS activation includes NE release from SNS nerve terminals and secretion of epinephrine (EPI) and NE from the adrenal medulla. Similarly, sAA levels also related to non-endocrine peripheral SNS markers under stress (Bosch et al., 2003; Nater et al., 2006). However, while an association between stress-induced SNS activation, particularly stress-induced NE secretion, and sAA release seems plausible, the mechanisms underlying this association are not fully understood (Bosch et al., 2011).

To date, the effect of NE-infusion on sAA release has been investigated in one animal study (Skov Olsen et al., 1988). In that pioneer study NE but also EPI were infused in 8 rats over a period of 3 h in supraphysiological dosage. Saliva was collected over several hours and sAA levels were assessed. Compared to control rats receiving saline-infusion only, NE- but also EPI-infusion elicited significantly higher sAA levels (Skov Olsen et al., 1988). With respect to observed associations between stress-induced NE and sAA release, results of that animal study may be interpreted in that stress-induced NE increases mediate (at least in part) sAA release (Skov Olsen et al., 1988). While in humans, a direct effect of NE- or EPI-infusion on sAA has not yet been investigated, studies using the non-selective beta-adrenergic agonist isoprenaline similarly observed a rise in sAA levels following isoprenaline infusion (Katz and Mandel, 1968; Speirs et al., 1974). Notably, to date no animal or human study has been conducted with an infusion procedure resembling in duration the NE-release evoked by acute laboratory stress (Nater et al., 2006).

Regarding receptor mechanisms underlying a potential NE-induced sAA increase following stress a mediating role of beta-adrenergic receptors seems evident: In a pharmacological pilot study Katz and Mandel (1968) administered isoprenaline in combination with the non-selective beta-adrenergic antagonist H56/28 in 5 men. Propranolol treatment inhibited isoprenaline-induced sAA increases. This early finding was confirmed in a series of pharmacological experiments in humans (Speirs et al., 1974; Nederfors and Dahlof, 1992, 1996; Nederfors et al., 1994). Similarly, in a stress study, prior propranolol administration reduced psychosocial stress-induced sAA increases (van Stegeren et al., 2006). To date, the role of *alpha-adrenergic* receptors in mediation of stress-induced sAA increases is still unclear. In the previously mentioned pioneer infusion study in rats blockade of alpha1- and alpha2-adrenergic receptors by phenoxybenzamine reduced EPI-infusion-induced sAA increases (Skov Olsen et al., 1988). Notably, in that study blockade of alpha-adrenergic receptors was associated with lower inhibition of EPI-infusion induced sAA release as compared to non-selective beta-adrenergic blockade by propranolol (Skov Olsen et al., 1988). In contrast, two human studies suggest stimulatory effects of alpha adrenergic blockade on sAA: in 5 men higher sAA increases had been observed following isoprenaline-infusion after alpha1- and alpha2-adrenergic receptor blockade by phentolamine (Katz and Mandel, 1968). Similarly, our group recently demonstrated in 13 men that a bolus-infusion of the alpha2-adrenergic receptor antagonist yohimbine significantly increased sAA as well as NE and EPI levels (Ehlert et al., 2006). However, it remains unclear whether this results from a central nervous and/or a peripheral effect of yohimbine and because of or despite alpha2-adrenergic (auto)receptor blockade (Goldberg and Robertson, 1983). In sum, given the reported associations between stress-induced sAA and NE increases (Chatterton et al., 1996; Rohleder et al., 2004; Thoma et al., 2012), and given the role of alpha-adrenergic mechanisms in sAA secretion in rats (Skov Olsen et al., 1988) and humans (Katz and Mandel, 1968; Ehlert et al., 2006) while taking into account that NE effects are primarily mediated by alpha-adrenergic receptors (Lees, 1981), alpha-adrenergic receptors may also be involved in mediation of stress-induced sAA increases in humans.

Here, we investigated for the first time in a placebo-controlled within-subjects design whether in healthy men NE-infusion induces sAA increases as commonly observed in reaction to acute psychosocial stress (Rohleder et al., 2006; Wirtz et al., 2009; Thoma et al., 2012), and whether these potential increases relate to alpha-adrenergic receptor mechanisms. We infused a NE-stress-reactivity mimicking dosage of NE with and without non-selective

alpha-adrenergic blockade by phentolamine and repeatedly measured sAA levels before and several times after infusion procedures. Successful phentolamine application was verified by investigating its known diastolic blood pressure (DBP)-decreasing (Richards et al., 1978), heart rate (HR)-increasing (Chatterjee and Parmley, 1977), and attenuating effects on NE-induced blood pressure (BP) increases (Carbonell et al., 1988). Based on Skov Olsen et al. (1988), we hypothesized NE-infusion to immediately increase sAA levels without but not with alpha-adrenergic blockade. Notably, we also investigated in the same experiment the influence of NE-infusion with and without non-selective alpha-adrenergic blockade by phentolamine on salivary cortisol levels (for details on rationale, methods, results, and discussion see Supplementary material).

2. Methods

2.1. Participants

The study is part of a larger project assessing effects of a NE-stress-reactivity mimicking NE-infusion with and without alpha-adrenergic blockade. The study sample for this part of the study comprised 21 medication-free, non-smoking healthy Caucasian men between 29 and 66 years of age who completed all trials with complete salivary measures and age-balanced trial-sequence. Participants were recruited with the aid of the Swiss Red Cross of the Canton of Bern and the Clinical Investigation Unit of the University Hospital of Bern/Inselspital. Specific exclusion criteria as verified in a clinical interview were: psychiatric diseases, any regular or acute medication intake, regular strenuous exercise, alcohol, smoking and illicit drug abuse, any heart disease, varicosis and thrombotic diseases, elevated blood sugar and diabetes, elevated cholesterol, liver and renal diseases, chronic obstructive pulmonary disease, allergies and atopic diathesis, rheumatic diseases, HIV, cancer, and current infectious diseases. Notably, none of our participants reported any problems with oral health. All participants provided written informed consent before any study procedure and were compensated with 120 CHF per day (total 3 days 360 CHF) for their participation.

The Ethics Committee of the Canton of Bern, Switzerland, and the Swiss Agency for Therapeutic Products (Swissmedic) formally approved the research protocol.

2.2. Design and procedure

The study was performed at the Clinical Investigation Unit of the University Hospital of Bern/Inselspital. In a single-blind placebo-controlled within subject design, all participants took part in three different experimental trials varying in terms of substance administration combination of a first infusion followed by a second infusion: trial-1 (placebo-plus-placebo, i.e., both infusions are physiological saline), trial-2 (placebo-plus-NE, i.e. the first infusion is placebo followed by NE as the second infusion), and trial-3 (blocker-plus-NE, i.e., the first infusion is the non-selective alpha-adrenergic blocker phentolamine followed by NE as the second infusion). Trial-1 was intended to control for the infusion procedure per se. Trial-2 was the main experimental trial intended to test for effects of NE-infusion. Trial-3 was intended to test whether potential NE-infusion effects are modulated by alpha1- and alpha2-adrenergic receptors or not and thus to test for underlying receptor mechanisms of NE-infusion. The trial-sequence was fully counterbalanced by using a Latin Square design applying the sequences (1,2,3; i.e., infusion day 1 was trial-1, infusion day 2 was trial-2, infusion day 3 was trial-3), (2,3,1; i.e., infusion day 1 was trial-2, infusion day 2 was trial-3, infusion day 3 was trial-1), and (3,1,2; i.e., infusion day 1 was trial-3, infusion day 2 was trial-1, and infusion day 3 was trial-2). Trials took place on separate days with inter-trial intervals of at least one week (to allow for a sufficient wash-out period of the alpha-adrenergic blocker phentolamine) up to two weeks. Participants were blind to the particular trial, and ethical considerations (e.g. potential side effects of study substances) precluded a double-blind design. All infusions were performed by a board-certified internist.

Participants abstained from physical exercise, alcohol, and caffeinated beverages from the evening before the test day, and maintained a regular sleep-wake rhythm the three nights before start of the study session, with sleep starting between 2230 h and 2400 h and ending between 0700 h and 0900 h. Participants reported to the laboratory at 1145 h to receive a standardized meal.

The experiment commenced at 1300 h. Participants were tested in supine position while lying on a bed. Each trial started with a 10 min introduction phase comprising an explanation of the testing procedure with subsequent catheter insertion into the brachial vein of the dominant-arm for substance-infusion. For blood sampling a second catheter was inserted into the brachial vein of the non-dominant-arm. The introduction phase was followed by a 45 min acclimation phase. Then, the infusion phase started. At the beginning of the infusion phase, placebo (or blocker) was infused for 1 min (first infusion), followed by a 5 min waiting interval. Next, NE (or placebo) was infused over a 15 min period (second infusion). Afterwards, a 150 min post-infusion phase began.

Stimulated saliva samples (by chewing on cotton roles for 1 min) for sAA determination were collected immediately before beginning of the infusion phase (baseline), after the first infusion and before beginning of the second infusion (between infusions), as well as 1, 10, and 20 min after the end of the second infusion. This sampling protocol was chosen based on previous stress studies showing that peak levels of sAA occur immediately after stress and return to baseline within the following 10 min (Nater et al., 2005; Het et al., 2009; Thoma et al., 2012). Blood samples for NE and EPI assessment were taken at baseline, and 1 min after the second infusion. In one participant NE and EPI levels of trial-1 were missing due to technical problems. Resting BP was measured 60 min and 5 min before beginning of infusion procedures. BP and HR assessment for verification of effective phentolamine application was performed by means of Omron sphygmomanometry (Omron 773, Omron Heathcare Europe B.V. Hoofdorp, Netherlands) immediately before beginning of the infusion phase (baseline), after the first infusion and before beginning of the second infusion (between infusions), as well as twice during (i.e., 6 min and 12 min after beginning of infusion 2) and 10, and 20 min after the second infusion. In one participant, HR for trial-3 was

missing due to technical problems. Self-reported state anxiety was measured at baseline and 7 min after beginning of the second infusion to assess a potential anxiety reaction to substance infusion. State anxiety levels were missing in one participant for trial-2, and in another participant for trial-3.

2.3. Substance injection

NE (norepinephrine, Sintetica, SA, Mendrisio, Switzerland) was diluted in physiological saline and adjusted to infuse 5 μg NE at a constant speed of 1 ml/min over a 15 min period (rendering a total of 75 μg NE) with an infusion pump. The NE-infusion dosage was chosen because of the following: First, prior findings showed that NE plasma levels in excess of 1800 pg/ml are required to produce measurable hemodynamic and/or metabolic effects as expected to occur in reaction to acute stress (Silverberg et al., 1978). Second, in a pilot study (data not shown) we tested this dosage (5 μg/min, in 5 healthy young men) as compared to a lower dosage (2.5 μg/min, in 4 healthy young men) and found the higher NE dosage to produce blood pressure increases that better mimic stress-induced blood pressure increases (Wirtz et al., 2006). The non-selective alpha-adrenergic blocker phentolamine (Regitin®, Novartis Pharma AG, Basel, Switzerland) was diluted in physiological saline and adjusted to infuse 2.5 mg in 5 ml within 1 min according to a pharmacologist's instruction based on the manufacturer's recommendation. In the first placebo-infusion, i.e. the placebo for the blocker, 5 ml of physiological saline were injected within 1 min. In the second placebo-infusion, i.e. the placebo for NE, physiological saline was injected at a constant speed of 1 ml/min over a 15 min period with an infusion pump. The 15 min infusion interval was chosen based on the duration of stress induction by means of the well-established and potent Trier Social Stress Test (TSST) (Kirschbaum et al., 1993) to make our NE-infusion in terms of duration resembling the NE release induced by acute psychosocial stress with the TSST.

2.4. Biochemical measures

Saliva samples for sAA assessment were collected in salivettes (Sarstedt, Sevelen, Switzerland) and stored at −20 °C until analysis in the Biochemical Laboratory, Institute of Psychology, University of Zurich. Centrifugation of thawed saliva samples was at 3000 rpm for 10 min, yielding low-viscosity saliva. sAA was measured by using a commercially available enzymatic colorimetric assay according to IFCC with a lower detection limit of 3 U/l (Roche diagnostics GmbH, Mannheim, Germany). Inter- and intra-assay coefficients of variance (CVs) were <10%.

For NE and EPI assessment, venous blood was drawn in EDTA-coated monovettes (Sarstedt, Numbrecht, Germany), and immediately centrifuged for 10 min at 2000 × *g* and 4 °C. Obtained plasma was stored at −80 °C until analysis. Plasma NE and EPI levels were determined in the Laboratory for Stress Monitoring, Göttingen, Germany by means of high-pressure liquid chromatography (HPLC) and electrochemical detection after liquid-liquid extraction (Smedes et al., 1982; Ehrenreich et al., 1997) with a detection limit of 12 pg/ml and inter- and intra-assay CVs < 5%. Plasma EPI levels below detection limit were replaced by detection limit divided by 2.

2.5. State anxiety

State anxiety was measured by self-report using the 20-item state version of the State-Trait Anxiety Inventory (STAI) (Laux et al., 1981). The state version assesses the level of anxious feelings at the moment. Items have a 4-point rating format reflecting level of feelings (ranging from 1 [not at all] to 4 [very much so]). Higher scores mean higher levels of state anxiety.

2.6. Statistical analyses

Data were analyzed using SPSS (version 20.0) statistical software package (SPSS Inc., Chicago IL, USA) and presented as mean ± SEM. All tests were two-tailed with the significance level set at $p \leq .05$ and the level of borderline significance set at $p \leq .10$. Normal distribution of data was verified using the Kolmogorov–Smirnov test prior to statistical analyses.

A-priori sample size calculation using G*Power 3.1 revealed that the optimal number of observations was $N = 60$ to detect trial differences in sAA levels of medium effect size ($f = .25$) in general linear models with repeated measures with 5 repetitions (sAA) that intercorrelate >.07 with a power of .90.

We calculated body mass index (BMI) as the weight in kilograms divided by height in meters squared. Mean arterial blood pressure (MAP) was calculated from resting blood pressure readings of infusion days 2 and 3 by the formula (2/3 mean DBP + 1/3 mean systolic BP, SBP).

Infusion-induced NE and EPI changes were calculated as the difference in plasma levels between 1 min post-infusion (second infusion) and baseline. Infusion-induced SBP, DBP, and HR changes were calculated by subtracting baseline levels from levels obtained 1 min after infusion 2. Infusion-induced changes in state anxiety were calculated by subtracting baseline scores from scores obtained 7 min after beginning of the second infusion.

To test for differences between trials (i.e. trial-1 vs. 2, trial-1 vs. 3, and trial-2 vs. 3) in baseline sAA measures, NE, EPI, and state anxiety baseline levels, and infusion-induced NE, EPI, and state anxiety changes, we calculated dependent *t*-tests.

To verify effective phentolamine application by investigating its known effects on BP and HR, we calculated general linear models with two repeated factors, trial (2 NE-trials, i.e. trial-2 and trial-3) and time (6 DBP/SBP/HR measurements). As post hoc tests, we calculated dependent *t*-tests to test for differences between trial-2 and trial-3 in SBP change, DBP change, and HR change, as well as between DBP and HR levels before and after infusion 1 (phentolamine or placebo).

To test the effects of the different infusion trials on sAA over time, we first calculated for each trial absolute sAA changes from baseline by subtracting the respective baseline level from all repeated sAA levels. We then calculated general linear models with two repeated factors, trial (3 trials) and time (5 sAA change measurement

time-points). As post hoc tests we compared trials pairwise (i.e. trial-1 vs. 2, trial-1 vs. 3, and trial-2 vs. 3) by calculating general linear models with the two repeated factors, trial (2 trials) and time (5 sAA change measurement time-points). Moreover, we tested whether sAA levels significantly changed over time in each trial by calculating general linear models with repeated salivary measures in each trial separately to obtain information regarding significance of main effects of time. As we decided against weight adjustment of infusion concentrations and in light of previously reported associations of cardiovascular risk factors and indicators for SNS activity (Strahler et al., 2010) we controlled for the cardiovascular risk factors BMI, age, and MAP in all repeated sAA analyses as an a-priori defined set of covariates.

Effect size parameters (f) were calculated from partial η^2-values and are reported where appropriate (effect size conventions: f: .10 = small, .25 = medium, .40 = large).

3. Results

3.1. Subjects' characteristics

Study participants were middle-aged to older (52.2 ± 2.39, range: 29–66 years) and non-obese (BMI: 24.0 ± .39, range: 21.7–29.0 kg/m^2) men. All participants were normotensive according to the obtained blood pressure readings (mean SBP: 117.7 ± 1.92, range: 104.8–134.5 mmHg, mean DBP: 73.2 ± 1.41, range: 62.3–84.3 mmHg, mean MAP: 88.0 ± 1.38, range: 76.6–98.3 mmHg). Participants of the 3 trial-sequences (1,2,3; 2,3,1; and 3,1,2) did not differ in age (trial-sequence 1,2,3: 50 ± 4.4 years; trial-sequence 2,3,1: 55 ± 3.6 years; trial-sequence 3,1,2: 51 ± 4.7, p's > .43).

3.2. Verification of effective phentolamine application

Successful phentolamine application was verified by comparing repeated HR and BP readings between NE-infusion trial-2 (NE-infusion without alpha-adrenergic blockade) and trial-3 (NE-infusion with alpha-adrenergic blockade). Infusion reactivity of SBP (interaction trial-by-time: $F(5/100) = 2.41$, $p = .041$, $f = .35$), DBP (interaction trial-by-time: $F(4.6/91.3) = 4.9$, $p = .001$, $f = .49$), and HR (interaction trial-by-time: $F(38.8, 95.0) = 2.51$, $p = .035$, $f = .36$) differed in the expected directions between trials (see Fig. 1A–C). Post hoc tests revealed that compared to NE-infusion without alpha-adrenergic blockade (trial-2), NE-infusion with alpha-adrenergic blockade (trial-3) induced smaller increases in SBP (SBP change: $t(20) = 2.06$, $p = .053$) and DBP (DBP change: $t(20) = 3.59$, $p = .002$), as well as reduced decreases in HR (HR change: $t(19) = -2.16$, $p = .043$). Moreover, in trial-3, phentolamine infusion immediately reduced DBP, and increased HR prior to NE-infusion (DBP: $t(20) = -6.51$, $p < .001$; HR: $t(19) = 2.87$, $p = .010$), while placebo-infusion-1 did not (DBP: trial-1: $t(20) = -.15$, $p = .88$; trial-2: $t(20) = -.26$, $p = .80$; HR: trial-1: $t(20) = -.70$, $p = .49$; trial-2: $t(20) = -.34$, $p = .74$).

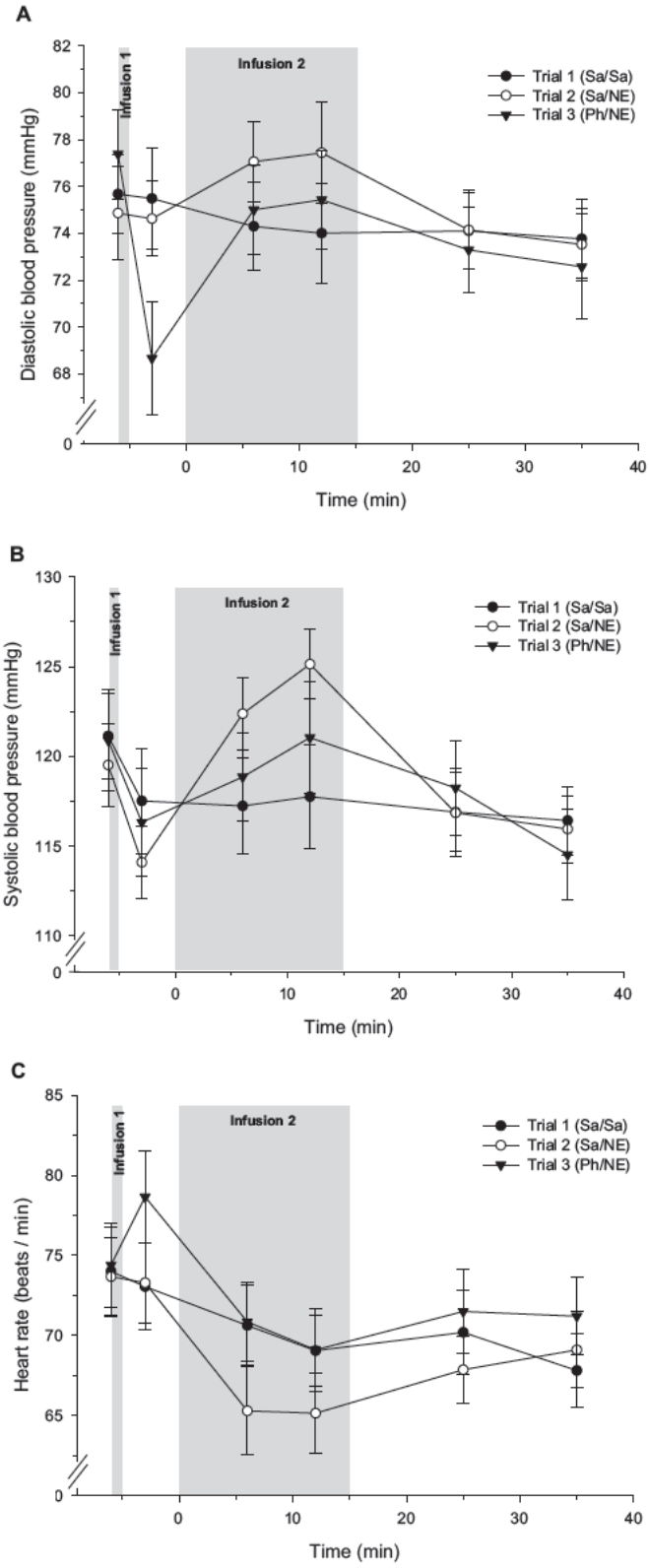

Fig. 1 Diastolic blood pressure (A), systolic blood pressure (B) and heart rate (C) reactivity to substance infusion. Values are means ± SEM. As expected, infusion reactivity of DBP ($p = .001$), SBP ($p = .041$), and HR ($p = .035$) differed between trial-2 and trial-3. Sa, saline; NE, norepinephrine; Ph, phentolamine.

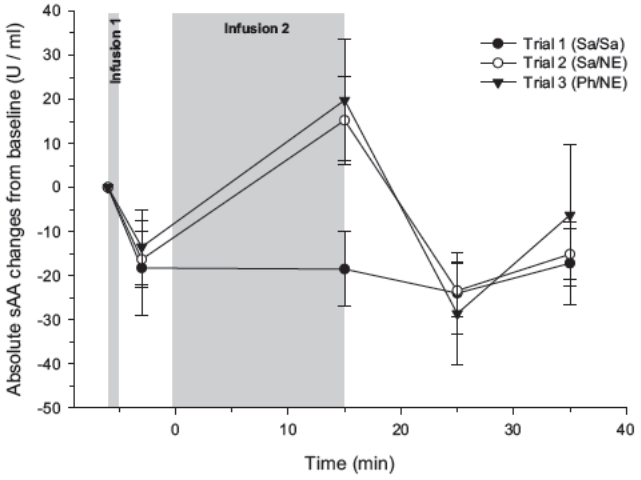

Fig. 2 Salivary alpha amylase (sAA) reactivity to substance infusion. Values are means ± SEM. Repeated measures ANCOVA revealed that experimental trials significantly differed in sAA reactivity to substance infusion ($p = .001$) with trials 2 and 3 significantly differing from trial 1 (p's $\leq .004$) Age, BMI, and MAP were controlled. Sa, saline; NE, norepinephrine; Ph, phentolamine.

3.3. Trial comparisons in baseline measures and infusion-induced changes in catecholamines and state anxiety

Table 1 shows that there were no baseline (i.e. pre-infusion) differences between the 3 trial conditions in plasma catecholamine (p's > .15) and state anxiety levels (p's > .08), as well as between trial-1 and trial-2 in sAA levels ($p = .31$). In contrast, baseline sAA levels were higher in trial-3 as compared to trial-2 ($t(20) = 2.80$, $p = .011$) and trial-1 ($t(20) = 2.17$, $p = .042$).

While saline-only infusion (trial-1) did not cause increases in NE plasma levels ($p = .59$), NE-infusion with (trial-3: $t(20) = 9.19$, $p < .001$) and without alpha-adrenergic blockade (trial-2: $t(20) = 7.17$, $p < .001$) did as expected. Moreover, none of the infusion conditions induced significant increases in EPI levels (trial-1: $p = .52$; trial-3: $p = .78$) or state anxiety scores (trial-1: $p = .11$: trial-2: $p = .76$; trial-3: $p = .47$). EPI levels actually decreased after NE-infusion without alpha-adrenergic blockade (trial-2: $t(20) = -4.49$, $p < .001$).

3.4. sAA measures in reaction to infusions 1 and 2 in the trial conditions

Experimental trials significantly differed in sAA reactivity to substance-infusion (interaction trials-by-time: $F(7.0/118.6) = 3.71$, $p = .001$, $f = .47$; see Fig. 2). Post hoc tests revealed higher sAA reactivity following NE-infusion with (trial-3; $F(4.0/68.0) = 5.46$, $p = .001$, $f = .57$) and without alpha-adrenergic-blockade (trial-2; $F(3.6/60.5) = 4.50$, $p = .004$, $f = .51$) as compared to saline only infusion (trial-1). sAA infusion reactivity was not different between trial-2 (NE-infusion without alpha-adrenergic blockade) and trial-3 (NE-infusion with alpha-adrenergic blockade) ($p = .29$). Further post hoc testing revealed that NE-infusion elicited

Table 1 Baseline measures and infusion-induced changes in norepinephrine (NE), epinephrine (EPI), and state anxiety.

	Trial 1 (Sa/Sa)	Trial 2 (Sa/NE)	Trial 3 (Ph/NE)	*t*-Tests		
				$p^{(1 \text{ vs. } 2)}$	$p^{(1 \text{ vs. } 3)}$	$p^{(2 \text{ vs. } 3)}$
sAA baseline (U/ml)	81.0 ± 17.36 (2.5–344.8)	66.1 ± 11.41 (6.2–196.8)	118.9 ± 23.31 (1.6–383.0)	.31	**.042**	**.011**
NE baseline (pg/ml)	433.4 ± 50.83 (190.2–976.5)	408.5 ± 50.50 (145.6–1097.5)	385.0 ± 37.7 (126.1–778.1)	.56	.25	.55
EPI baseline (pg/ml)	29.8 ± 3.85 (6.0–66.01)	30.7 ± 3.47 (6.0–58.65)	27.0 ± 3.07 (6.0–27.03)	.85	.45	.16
State anxiety baseline	26 ± 1.22 (20–39)	28 ± 1.6 (20–50)	26 ± 1.1 (20–34)	.082	.71	.52
NE change (pg/ml)	9.6 ± 17.45 (−142.1–282.0)	782.1 ± 101.40 (138.6–1858.2)	757.5 ± 82.4 (187.8–1661.9)	**<.001**	**<.001**	.77
EPI change (pg/ml)	1.6 ± 2.46 (−15.4–37.5)	−5.9 ± 1.31 (−16.4–11.2)	−.7 ± 2.31 (−10.2–36.4)	**.004**	.50	**.013**
State anxiety change	−1.2 ± .74 (−11–4)	−.7 ± 2.07 (−25–24)	.6 ± .82 (−4–14)	.78	.17	.66

Notes: Values are given as means ± SEM (range). Dependent *t*-tests were conducted to test for trial differences. Bold values indicate significance. Sa, saline; Ph, phentolamine; sAA, salivary alpha amylase.

significant increases in sAA over time (trial-2: main effect of time: $F(3.0/51.4) = 4.76$, $p = .005$, $f = .53$; trial-3: main effect of time: $F(3.6/61.7) = 7.46$, $p < .001$, $f = .66$) while saline-infusion related to significant sAA decreases (trial-1: $F(3.8/64.7) = 6.44$, $p < .001$, $f = .62$). Results remained significant without controlling for age, BMI, and MAP except that sAA decreases after saline-infusion became of borderline significance (trial-1: $p = .095$).

3.5. sAA reactivity to infusion 1 (saline or phentolamine) in the trial conditions

While sAA levels decreased significantly following saline-infusion in trial-1 ($F(1.0/17.0) = 9.22$, $p = .007$, $f = .74$), no significant sAA changes were observed following both saline-infusion in trial-2 and phentolamine-infusion in trial-3 (p's > .24).

4. Discussion

This is the first placebo-controlled study that systematically investigates within a sufficient sample size of healthy men underlying mechanisms of sAA increases as usually observed in reaction to acute psychosocial stress by infusion of a NE-stress-reactivity mimicking NE dosage with and without alpha-adrenergic blockade by phentolamine. Notably, our NE-infusion dosage induces comparable blood pressure increases as psychosocial stress does and mimics the duration of NE release during acute laboratory stress induction (by means of the TSST). As hypothesized, we found pronounced increases in sAA levels immediately after completion of NE-infusion as compared to placebo. In contrast to our hypothesis, non-selective alpha1- and alpha2-adrenergic receptor blockade by phentolamine did not alter NE-induced sAA increases as evidenced by the lack of an inhibitory effect of phentolamine on NE-induced sAA changes. We also did not observe significant changes in sAA levels from immediately before to immediately after phentolamine-infusion. In relation to the high but normal sAA baseline variation (e.g. Nater et al., 2007) our observed NE-infusion-induced sAA increases may appear modest but were of large effect size and independent of BMI, MAP, and age.

Our finding of increased sAA levels in response to a NE-stress-reactivity mimicking NE dosage is consistent with results from Skov Olsen et al., (1988) who also observed increased sAA levels after infusion of a supraphysiological NE dosage in rats. Furthermore, absolute NE-induced sAA increases (sAA change from immediately before to immediately after infusion 2) observed in this study correspond well with those sAA increases (sAA change from immediately before to immediately stress) observed in response to acute psychosocial stress under comparable saliva sampling time and saliva collecting conditions (Nater et al., 2006; Rohleder et al., 2006; Strahler et al., 2010). Our data thus suggest that the mechanism underlying stress-induced sAA increases is likely to involve NE. Given that stress induces immediate NE increases (e.g. Nater et al., 2006) we interpret our data in that stress-induced NE-increases are capable of inducing sAA increases. Such reasoning is in line with previous human stress studies reporting associations between stress-induced plasma NE and sAA increases (Chatterton et al., 1996; Rohleder et al., 2004; Thoma et al., 2012). However, it should be kept in mind that our peripheral infusion procedure clearly differs from psychosocial stress induction. Consequently, we can only speculate whether our findings apply to NE-stress-reactivity in reaction to the complex physiological process of psychosocial stress induction.

Regarding mechanisms underlying NE-infusion induced sAA increases, our study is the first to investigate whether alpha-adrenergic receptors may mediate NE-induced sAA increases. We found no effect of the non-selective alpha-adrenergic antagonist phentolamine on NE-induced sAA increases. Furthermore, phentolamine-induced blockade of alpha-adrenergic receptors prior to NE-infusion did not change sAA secretion. The HR-increasing effect, as well as the antihypertensive effects of phentolamine and thus its successful application suggest, at first glance, that alpha1- and alpha2-adrenergic receptors are not involved in mediating sAA secretion following NE-infusion or endogenous NE increases, respectively. This, however, does not correspond with the three hitherto published animal (Skov Olsen et al., 1988) and human (Katz and Mandel, 1968; Ehlert et al., 2006) studies suggesting an alpha-adrenergic receptor involvement in sAA secretion. Several methodological reasons may contribute to our non-significant phentolamine findings and thus to the apparent inconsistency with the literature: While we infused phentolamine (non-selective alpha-adrenergic antagonist) combined with NE (alpha- and beta-adrenergic agonist), Katz and Mandel infused phentolamine combined with isoprenaline (beta-adrenergic agonist), Skov Olsen et al. infused phenoxybenzamine (non-selective alpha-adrenergic antagonist) combined with EPI (alpha- and beta-adrenergic agonist), and Ehlert et al. infused yohimbine (alpha2-selective adrenergic antagonist) without additional infusion. Given that these pharmacological agents not only differ in their target receptor type, but also vary in their binding affinity to their respective target receptor, and given that different concentrations of these agents were used, inconsistent findings are not surprising. Moreover, it should be taken into account, that results from animal studies are not necessarily applicable to humans (Katz and Mandel, 1968). Besides methodological differences, interpretational issues may also account for our non-significant phentolamine findings and the inconsistency of our findings with previous data. On the one hand, potential opposing peripheral and/or central effects of phentolamine-induced blockade of alpha-1 vs. alpha-2 adrenergic receptors on NE-induced sAA release cannot be excluded (e.g. Ehlert et al., 2006). Notably, potential direct central effects are not very likely given that phentolamine is considered unable to cross the blood brain barrier (Nordling et al., 1981). On the other hand, despite promising animal data (Bowser-Riley et al., 1978; Sayardoust and Ekstrom, 2006), it has not yet been investigated in humans whether phentolamine easily diffuses from intravenous fluid into the interstitial glandular tissue to activate the parenchyma secretory cells. Given this uncertainty, our non-significant effect of alpha-adrenergic blockade on NE-infusion induced sAA release needs to be interpreted with caution. Notwithstanding this, it seems likely to assume, in line with previous research (Katz and Mandel, 1968; Speirs et al., 1974; Skov Olsen et al., 1988; Nederfors and Dahlof, 1992, 1996; Nederfors et al., 1994; van Stegeren et al.,

2006), that beta-adrenergic receptors mediate NE-infusion induced sAA increases.

The major strengths of our study were the choice for a NE-infusion design that mimics NE-stress-reactivity effects in terms of duration and dosage, an appropriate sample size, and the chosen saliva sampling-time point protocol for sAA determination, which allowed us to also investigate the effects of phentolamine (non-selective alpha-adrenergic antagonist) on sAA secretion without NE-induced sAA stimulation. A further strength of our study was the state-of-the-art use of salivette saliva collection devices with the standardized instruction to chew the synthetic swab for 1 min as this sampling method nearly rules out potential confounding effects by standardizing salivary flow rate (Rohleder et al., 2006). Furthermore, potential confounding factors were controlled in our statistical analyses by a priori entering age, BMI, and MAP as covariates. Moreover, we assessed self-reported state anxiety as well as the stress-sensitive SNS measures EPI and HR in addition to NE. The lack of an increase in state anxiety, EPI, and HR following NE-infusion allowed us to conclude that the sAA increases observed in our study result from NE-infusion rather than from potential NE-infusion-induced arousal or stress responses. A final strength refers to the fact that we can exclude EPI as a confounding factor in NE-induced sAA changes as demonstrated by the lack of increases in EPI plasma levels immediately after NE-infusion.

Our study also has limitations. First, our findings are restricted to healthy men. Further studies are needed to replicate our findings in women or other populations. Second, it is unclear why higher baseline sAA levels were found in trial-3 compared to trial-2 and trial-1. However, to account for baseline differences between trials, we statistically analyzed sAA changes instead of sAA raw data. Third, we infused the same NE-dosage in all participants, which can increase inter-individual variability of dependent variables and thus the risk of non-significant findings. However, since we controlled for BMI as a kind of post hoc method to correct for weight differences and since we used a within-subject design, we feel that the risk of non-significant findings due to our infusion procedure is acceptable. Fourth, the clinical relevance of our observed sAA increases remains to be investigated in future studies. Fifth, our non-significant effects of alpha-adrenergic blockade on sAA release need to be interpreted with caution. Future studies are needed to clarify the role of alpha1- and alpha2-adrenergic receptor mechanisms in NE-infusion-induced and/or stress-induced sAA increases in more detail.

In sum, our findings indicate that sAA levels are directly stimulated by a NE-stress-reactivity mimicking NE-infusion with and without non-selective alpha-adrenergic blockade by phentolamine suggesting that the mechanism underlying stress-induced sAA increases may involve NE.

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Conflicts of interest statement

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2014.07.023.

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