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SHORT COMMUNICATION

Salivary alpha amylase as marker for adrenergic activity during stress: Effect of betablockade

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Summary Free salivary cortisol is an established non-invasive marker of hypothalamus pituitary adrenal (HPA) axis activity. In contrast, such a well-characterized salivary marker for activity of the sympatho-adrenal medullar (SAM) system is still missing. As one potential candidate salivary alpha amylase (sAA) has been suggested. In humans increases in sAA levels have been observed in response to physiological and psychological stress. The present study aimed at exploring the effects of a pharmacological manipulation (betablockade) on sAA in the context of a stressful fMRI experiment on emotional information processing. Thirty young healthy subjects participated in a double blind group comparison study and received 80 mg of the betablocker (BB) propranolol or a placebo (PL). Salivary samples were obtained before and 90 min (pre-scan) and 135 min (post-scan) after drug application. In addition heart rate and blood pressure were assessed. During rest a significant drug by time interaction was observed, lowering sAA levels as well as heart rate and systolic blood pressure in the betablocker treatment group. During the scanning procedure, in which participants were confronted with highly negative emotional pictures, the significant increase in sAA levels in the PL group compared to the BB group persisted. No additional change was noticed in heart rate or blood pressure during scanning in the PL or BB group. The current pharmacological study in the human provides direct evidence for the sensitivity of sAA to changes in adrenergic activation, specifically in reaction to psychological stress.

1. Introduction

The use of salivary sampling as a noninvasive tool for the assessment of free cortisol and therewith as a marker for activity of the hypothalamic pituitary

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adrenal (HPA) axis is well established in human stress research (Kirschbaum and Hellhammer, 1994). A corresponding relatively easy to obtain marker for the sympatho-adrenal medullar activity is still missing. Direct measurements of adrenaline and noradrenaline out of saliva seem not to reflect SAM activity (e.g. Schwab et al., 1992).

In this paper we will refer to (nor)adrenaline[1] as transmitter released by the sympathetic nerve endings as well as the peripherally acting hormones released by the adrenal medulla. The salivary gland and glandular duct cells as well as the vascular bed of the salivary glands are abundantly provided with beta-adrenoceptors (Nederfors and Dahlof, 1992). Adrenaline affects the activity of the parotid salivary glands also via non-innervated beta-receptors, that are therefore responsive to hormonal actions (Lovallo and Thomas, 2000).

Salivary alpha amylase (sAA) levels have been suggested as a potential indirect marker for SAM activity but additional research in this area is clearly warranted. Animal studies have suggested that sAA is secreted after beta-adrenergic stimulation (Gallacher and Petersen, 1983). Other studies found that the result of infusion of alpha- and beta-adrenergic antagonists as well as noradrenaline and isoproterenol showed that secretion of salivary amylase is predominantly mediated by stimulation of beta-adrenergic receptors (Skov Olsen et al., 1988). In humans sAA levels have been reported to rise in response to physical stress (Gilman et al., 1979; Chatterton et al., 1996) as well as to psychological stressors (Bosch et al., 1996; Chatterton et al., 1996; Nater et al., 2005). Moreover, one study reported that sAA levels were significantly associated with noradrenaline levels measured out of plasma samples (Chatterton et al., 1996). They conclude that salivary alpha-amylase concentrations are predictive of plasma catecholamine levels under a variety of stressful conditions.

An alternative and potentially more direct assessment on the impact of changes in adrenergic activity on sAA levels could be achieved by using beta-receptor agonists or antagonists to further validate the usefulness of sAA as an adrenergic marker. Indeed an early and very small pilot study ($n=5$) observed that 30 mg of the betablocker propranolol reduced sAA levels in some of the subjects (Speirs et al., 1974). In a series of experiments Nederfors (Nederfors and Dahlof, 1992; Nederfors et al., 1994; Nederfors, 1996) studied the effects of beta-adrenoceptor antagonists on saliva flow rate and composition. This was evaluated both in healthy volunteers and in hypertensive patients. The effects of 1 week of treatment with the non-selective (propranolol, 80 mg) and the beta 1-selective (atenolol, 50 mg) adrenoceptor antagonists were compared with that of placebo in three different clinical trials. Salivary composition but not saliva flow rates were affected by the beta-adrenoceptor antagonists (Nederfors, 1996).

The potential of alpha amylase as a salivary marker of adrenergic activity could be of substantial interest for human stress research since it would allow the parallel investigation of the two major neuro-endocrine stress systems with salivary samples (Chatterton et al., 1996).

The goal of the present placebo controlled double blind study therefore was to investigate the effects of a pharmacological manipulation of the SAM system when participants underwent a stressful procedure (watching highly negative emotional pictures in a scanner). For this purpose we used a non-selective betablocker (80 mg propranolol) to explore its effect on sAA levels and on two additional well-established markers of adrenergic activity (heart rate and blood pressure). We hypothesized first that betablockade would lower all markers of the SAM system during rest. Secondly, if increased sAA levels are a sensitive indicator of increased adrenergic activation, then the experimental procedure would have to provoke rising sAA levels under placebo condition, but less so under betablockade.

2. Method

2.1. Subjects

Thirty right-handed subjects (15 males, 15 females; mean age 20.93 ± 2.38, ranging from 18 to 28 years) without medical or psychiatric history were selected after an introduction interview, where they were screened with the Symptom Check List (SCL-90) (mean score $= 104.03 \pm 9.48$) and a biographic questionnaire. Subjects were students of the University of Amsterdam and received course credit for participation. The Medical Ethical Committee of the VU Medical Center (VUMC) approved the experiment and informed consent was obtained from all subjects. Data of 1 male and 1 female subject were excluded because no valuable measurements could be obtained from their saliva samples, possibly reflecting sample contamination.

[1] For the purpose of readability we use 'adrenaline' throughout the article to refer to the peripherally acting hormones adrenaline as well as noradrenaline.

2.2. Design and procedure

In this study we used a randomised, double blind, placebo-controlled design. Subjects came to the experimenter's room to participate in a comprehensive fMRI experiment. The data of that experiment are published elsewhere (van Stegeren et al., 2005). Only the first part of this experiment and the data pertaining to sAA measurements will be reported here. After an acclimatization period of 15 min, heart rate and blood pressure were measured for baseline ($t0$) values. Then an unstimulated saliva sample was taken for amylase measurements. Participants then received double blind either a placebo (PL) or betablocker (BB). Drugs were divided evenly over the sexes. A resting period of 90 min was needed to have the drug reach peak plasma levels (Gilman and Goodman, 1996). During this period participants were informed about the rest of the experimental procedure of that day (during approximately 10 min), and were then allowed to read a book or magazines, that they had been asked to bring with them. After that period the same measurements carried out at baseline ($t0$) were repeated just before ($+90$ min $=t1$) and immediately after the scanning procedure ($+135$ min $=t2$). During the scanning procedure—that took about 45 min—subjects were randomly presented with 92 stimulus pictures, varying from neutral scenes of domestic objects to extremely negative scenes of mutilation or accidents. After each picture subjects were asked on screen (within 3 s) to indicate the emotional intensity of the previous picture on a 4-points scale ranging from 1 ('not emotional at all') to 4 ('extremely emotional'). Off-line analysis indicated that the intended emotional induction by the stimulus material was successful (van Stegeren et al., 2005).

2.3. Cardiovascular measures

Cardiovascular reactions were registered with heart rate (HR) and blood pressure (systolic blood pressure = SYS; diastolic blood pressure = DIAS). Heart rate and blood pressure were measured with an Omron device (OMRON-IC, Healthcare Europe BV, Hoofddorp, The Netherlands), with a cuff applied around the left upper arm, when subjects were sitting in the room next to the scanner.

2.4. Amylase level

Salivary Alpha Amylase (sAA) levels were assessed out of unstimulated saliva samples obtained using regular cotton Salivette sampling devices (Sarstedt, Nümbrecht, Germany) without chemical stimulants. Subjects were instructed to just place the swab in their mouths for 30 s. After removal the salivettes were kept in the room and were stored at $-20\,^{\circ}C$ immediately after each subject's study protocol. Upon completion of the study (that took about 3 months), all samples were sent to Dresden for determination of amylase activity. Salivettes are centrifuged for 10 min at $2000\times g$ to obtain clear saliva. Alpha-Amylase is measured by a quantitative enzyme kinetic method. Saliva is diluted 1:625 with double-distilled water. 20 μl of diluted saliva and standard are transferred into transparent 96-well microplates (Roth, Germany) in duplicates. Standard is prepared from 'Calibrator f. a.s.' solution (Roche Diagnostics, Mannheim, Germany) ranging from 5.01 to 326 U/l Amylase, and double-distilled water as zero standard. After that, 80 μl of substrate reagent (Alpha -Amylase EPS Sys; Roche Diagnostics) are added. The microplate is then warmed to 37 °C in a water bath for 90 s. After a first interference measurement at 405 nm using a standard ELISA-reader (Anthos HT2, Anthos, Krefeld, Germany), the plate is incubated for another 5 min at 37 °C, and the second measurement is done. Increases of absorbance of samples are transformed to sAA concentrations using a linear regression calculated for the standard curve on each microplate (GraphPad Prism 4.0b for MacOSX, GraphPad Software, San Diego, USA).

2.5. Pharmacological treatment

The betablocker used was the non-selective centrally and peripherally acting beta1-and beta2-receptor blocker propranolol (80 mg, Centrapharm, Belgium) and as a placebo a similar looking pill was supplied (Albochin, Pharmachemie, The Netherlands). Both pills were prepared in the VUMC Pharmacological Department.

2.6. Statistical analysis

An ANOVA, general linear model (GLM) was used with the three time points ($t0$, $t1$ and $t2$) in the experiment as within subjects repeated measures and with the drug manipulation (BB versus PL) as between subject variable. T-tests were used to compare drug groups at the three time points.

3. Results

3.1. Effects of betablockade

At baseline (t0) drug groups were comparable with respect to sAA, HR, SYS and DIAS values

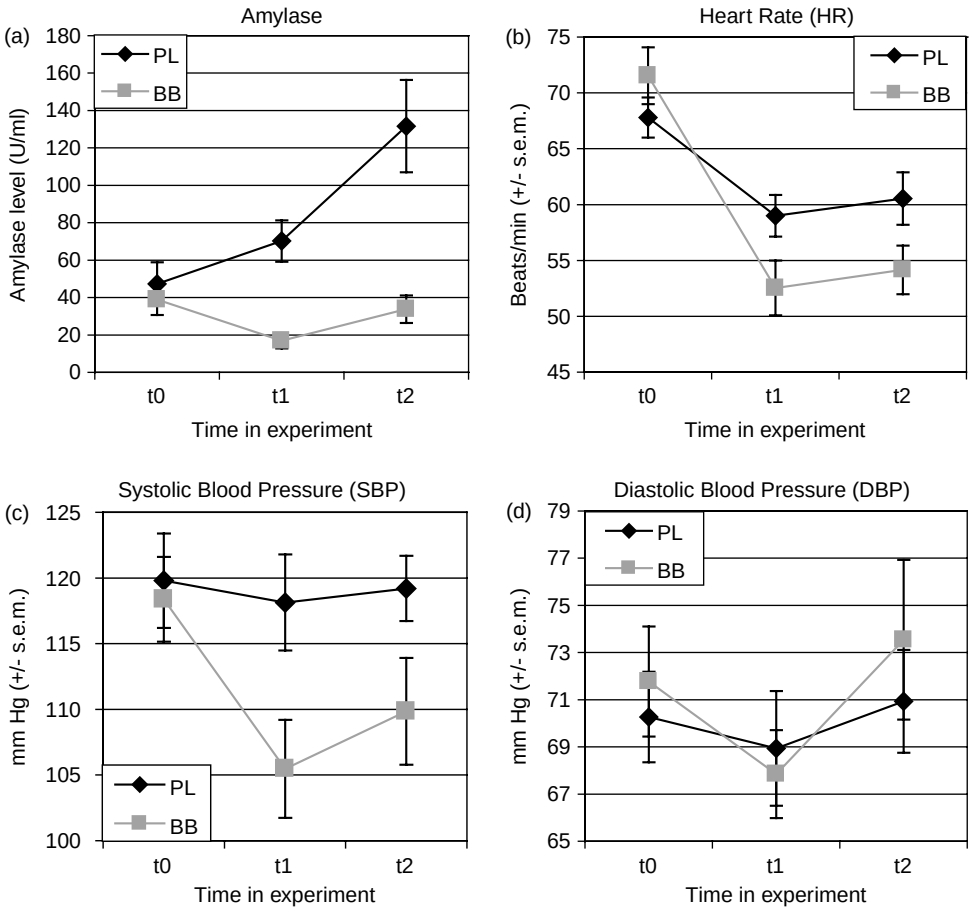

Figure 1 The effect of betablockade (80 mg propranolol) compared to placebo at baseline (*t*0), 90 min later pre-scan (*t*1) and immediately post-scan (*t*2) on Amylase (a), Heart rate (b), Systolic Blood pressure (c) and Diastolic blood pressure (d).

(all $p>0.10$). Then the drug (BB/PL) $\times$time (3) interaction effect on amylase was analysed. A significant interaction effect of drug with time ($F(2, 25)=11.76$, $p<0.001$) was found indicating that betablockade lowered the amylase level compared to placebo (Fig. 1a). Simple contrasts showed that sAA levels decreased significantly between *t*0 and *t*1 in the BB group, whereas sAA levels increased in the PL group ($F(1,26)=9.44$; $p<0.005$). Amylase levels in the PL group kept on rising throughout the scanning procedure, with the BB group showing only a small rise in sAA, resulting in a significant difference between the drug groups at t2 ($t(1,26)=3.57$; $p<0.002$) and a significant contrast of drug$\times$time between t0 and t2 ($F(1,26)=15.11$; $p<0.001$). The results obtained for HR and SYS were similar, with an interaction effect of drug$\times$time (HR: $F(2,25)=7.50$, $p<0.01$; SYS:$F(2,25)=6.47$, $p<0.005$) with significant repeated contrasts between t0 and t1 (HR: $p<0.001$; SYS: $p<0.001$) but not between t1 and t2 (for HR and SYS both $p>0.10$). So, betablockade was successful in decreasing HR and SYS compared to placebo and to keep this decrease throughout the complete study protocol (Fig. 1b and c). The effect of betablockade on diastolic blood pressure was not significant compared to placebo (1d). These interactions of drug x time on sAA, HR and SYS were even stronger when time of day (test time) and gender were used as a covariate. However no main effect of test time and gender were found on any of the dependent variables (data not shown).

4. Discussion

The main finding of the present study is that treatment with the betablocker propranolol blocked the stress induced increase of sAA levels observed in the placebo group. This observation extends previous work in animals (Gallacher and Petersen, 1983) and humans (Speirs et al., 1974; Nederfors and Dahlof, 1992; Nederfors et al., 1994; Nederfors, 1996) that showed that betablockade lowered sAA levels studied in a resting condition. Our

pharmacological experiment therefore strongly supports the notion that sAA levels reflect beta-adrenergic activity in the human. We observed a small rise in sAA levels between t0 and t1 in the placebo group, which most likely reflected some anticipatory anxiety caused by the upcoming fMRI investigation. Interestingly we did not observe such a rise for the other cardiac markers obtained, which could suggest that sAA is more sensitive to subtle psychological stress than blood pressure or heart rate. Alternatively this rise could reflect the normal circadian rhythm of sAA, which is characterized by a continuous increase over the course of the day (Rohleder et al., 2004). Controlling for test time however revealed that this increase was not depending on the circadian rhythm of sAA, but should be explained as a reaction to the procedure subjects were in, hence anticipatory psychological stress. This rise in sAA was completely abolished by the betablocker.

The scanning procedure was successful in evoking an emotional reaction (van Stegeren et al., 2005) marked by a significant increase in sAA levels, but not in heart rate or blood pressure. This can be explained by the fact that subjects had been lying in supine position in the scanner for approximately 45 min and had only been confronted with emotional visual stimuli without any physical exertion. Propranolol completely blocked this increase in sAA level, supporting the main hypothesis of this study.

Taken together the present pharmacological study in the human provides direct evidence for the sensitivity of sAA to changes in adrenergic activity and specifically as a marker in reaction to psychological stress. It can be concluded that, compared to HR and SYS, amylase is a more sensitive measure of sympathetic drive. This non-invasive easy obtainable marker therefore appears to be of interest for future human neuro-endocrine stress studies. Clearly additional methodological research is needed for a better understanding of the advantages and disadvantages of sAA levels in comparison to already established markers of SAM system activity.

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