The assessment of cortisol using salivary ultrafiltrate

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Cortisol can be used to indicate stress level as well as to monitor certain disease states. Although cortisol can be sampled from blood and urine, saliva sampling has decided advantages. Unfortunately, whole saliva contains a number of substances that can metabolize or bind with cortisol, potentially confounding analysis and quantitation. We introduce a semipermeable pouch that accumulates a contaminant-free salivary ultrafiltrate, thereby overcoming a number of the problems encountered with whole saliva. In three studies, we demonstrate accuracy and utility of the device for cortisol determination: (1) in an artificial medium, 60%–77% of the cortisol was recovered in the ultrafiltrate; (2) in vivo, ultrafiltrate cortisol correlated highly with whole-saliva cortisol collected under ideal conditions; and (3) ultrafiltrate cortisol evinced positive relationships with depression and cigarette use, consistent with studies in the literature. We conclude that this device and saliva-filtering technologies in general are useful in applications requiring quantitation of cortisol.

Behavioral researchers increasingly are quantitating biologically active molecules such as hormones, drugs and their metabolites, and immune markers. The steroid hormone cortisol has been the subject of much inquiry. Cortisol is an end product of hypothalamic–pituitary–adrenal (HPA) axis activity; it is released from the adrenal cortex following stimulation by pituitary adrenocorticotrophic hormone (ACTH), regulated in turn by hypothalamic corticotropin releasing factor (CRF). Cortisol is relevant to psychosocial research because the HPA axis plays a major role in the stress response. Cortisol provides an objective marker for stress because it increases reliably and linearly (within certain limits) in response to a wide range of physical and psychosocial stressors, ranging from laboratory stress to dominance hierarchies among primates (Levine, Coe, & Wiener, 1989; Linden, Dadgar, & Earle, 1994; Munck, Guyre, & Holbrook, 1984; Sapolsky, 1990).

Cortisol can be used as a marker of various disease states and health processes. Under certain conditions, cortisol decreases immunosurveillance, thus linking stress with infectious disease and cancer (Cohen & Williamson, 1991; Meuleman & Katz, 1985). Dysregulation of the HPA axis occurs in depression, and a lack of cortisol suppression following administration of the synthetic steroid, dexamethasone, has both diagnostic and treatment implications (Arana & Mossman, 1988; Ribeiro, Tandon, Grunhaus, & Greden, 1993). Excessive cortisol levels may impair certain cognitive functions (Wolkowitz et al., 1990), and cortisol may alter body fat distribution and adversely affect cardiovascular health (Rodin, 1992). Cortisol levels increase in response to nicotine administration, which may influence nicotine sensitivity and subsequent cigarette smoking (Pomerleau & Pomerleau, 1990a, 1990b).

Reliable and efficient quantitation of cortisol is useful. Although blood has been the traditional medium for cortisol assessment, it poses several disadvantages. Venipuncture must be conducted, requiring trained technicians. Venipuncture discourages some subjects from participating, elicits fear and/or fainting in others, and renders research with children, unsupervised field studies, and multiple assessments across time difficult or impossible. Repeated blood sampling for cortisol is particularly problematic, because the pain and/or stress of venipuncture

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elicits temporary cortisol elevations, necessitating prolonged adaptation periods. Finally, 90% to 95% of cortisol in the blood is reversibly bound to corticosteroid-binding globulin (CBG) proteins; although only the free (unbound) fraction of cortisol is physiologically active, most assays quantitate the total cortisol concentration. Urine samples also have been employed. Urine's principle advantage is the ability to accumulate cortisol secretion over an extended period; among the disadvantages are sampling and esthetics as well as the inability to pinpoint episodic stimulation of cortisol.

Saliva is an alternative to blood and urine sampling because many blood-borne molecules readily enter saliva via intercellular diffusion through the lipid bilayer of the acinar cells of the salivary gland. Only the free fraction of steroids such as cortisol diffuses through the cells, and concentrations are independent of salivary flow rate; thus, saliva concentrations are directly proportional to—though significantly lower than—plasma concentrations. The validity of saliva determination for a range of substances, including cortisol, is well recognized (Ferguson, 1984; Schramm, Kidwell, Smith, & Craig, 1992; Tenovuo, 1989; Vining & McGinley, 1986, 1987). Additionally, saliva collection obviates the many logistic and measurement reliability problems associated with venipuncture and blood sampling. For these reasons, salivary assessment of cortisol has been advocated as the preferred method (Kirschbaum & Hellhammer, 1989).

Saliva assessment of cortisol poses unique problems, however. The usual saliva-sampling method requires repeated expectoration until sufficient whole saliva has been accumulated. Some subjects are disturbed by "spitting" into a tube and viewing their saliva; others experience "dry mouth." The time required to accumulate sufficient saliva varies among subjects, from several seconds to many minutes. Techniques sometimes employed by investigators to overcome dry mouth, such as chewing on an object such as parafilm or a rubber band, or even imagining biting a lemon, have unknown effects on cortisol secretion. Finally, expectoration requires motor activity by the subject, thus interrupting the ongoing stream of behavior.

More important, perhaps, is that whole saliva contains many components that may affect saliva processing and analysis. Oral squames, mucopolysaccharides, and mucoproteins contribute to saliva's highly viscous, sticky consistency, making laboratory handling (centrifugation and pipetting) laborious and inaccurate. Whole saliva contains substances that may affect the measured concentration of cortisol. Enzymes from food, oral microorganisms, or endothelium cells from the salivary gland may metabolize steroids (Laudat et al., 1988; McCracken & Poland, 1989; Meulenberg & Hofman, 1990). Although cooling or freezing of the saliva sample may slow or halt enzymatic metabolism, in practice, whole saliva often is subjected to enzymatic degradation because it is left at room temperature for extended and variable periods. Other saliva components can bind cortisol, resulting

in erroneous estimates of the free fraction. For example, red blood cells and other blood proteins, which are found in 20%–50% of saliva samples (Piazza et al., 1989), may bind cortisol (Hiramatsu & Nisula, 1988), as may food protein residues and gingival and mucosal exudates, which increase after toothbrushing (Schramm, Smith, Craig, & Grates, 1993).

It is desirable to improve saliva collection procedures and minimize sources of processing difficulty and measurement error. A number of technologies have been developed to accomplish these goals, and it is the objective of this paper to introduce to a behavioral audience an ultrafiltrate device—an osmotic collector that consists of a small pouch made from a semipermeable membrane. The pouch is held in the mouth and collects an ultrafiltrate of saliva that is free of components that interfere with ease of handling and accurate cortisol measurement (Schramm, Annesley, Siegel, Sackellares, & Smith, 1991). Supporting data are presented from three studies: two evaluate reliability and accuracy of the device in collecting cortisol from an artificial medium and from human saliva; the third demonstrates the device's utility to predict individual differences in depression and smoking. In the Discussion section below, saliva-filtering technologies will be examined and compared.

STUDY 1

Accuracy of the Device In Vitro

The first study was conducted using an artificial saliva medium to determine (1) the rate and extent of cortisol transfer into the osmotic collector, and (2) the degree of binding of cortisol to the device's external surface.

Method

Ultrafiltrate collection devices. The saliva collection devices were made from semipermeable membranes (regenerated cellulose) designed for hemodialysis (Cuprophane; Enka AG, Germany). Two membrane disks (35 mm in diameter and 20 μ m thick) were bonded with polyurethane (Tycel; Lord Corporation, Erie, PA) by applying a 5-mm-wide adhesive ring at the periphery and pressing the disks together. The membrane pores have an average molecular cutoff of 12,000 daltons, preventing entry into the pouch of relatively large molecules such as enzymes, mucopolysaccharides, and blood proteins, but allowing cortisol and other small molecules to pass through. Salivary flow is stimulated by two means: the pouch contains granular sucrose (0.75 g), which elicits salivation, and the pouch is moved around with the tongue. The pouch accumulates a volume of 1–1.5 ml of salivary ultrafiltrate via osmosis. Because large molecules are excluded, the ultrafiltrate has low viscosity (less than that of serum) and can be processed without centrifugation (Schramm et al., 1991).

Reagents. Human serum albumin, alpha-amylase (type VIII-A from barley malt), lysozyme (grade III), mucin (type I), and cortisol were purchased from the Sigma Chemical Co., St. Louis. Human gamma globulin (IgG) and scintillation cocktail, CytoScint ES, were purchased from ICN Biomedicals, Irvine, CA. Radio-labeled ^3H -cortisol ([1,2- ^3H (N)]-hydrocortisone, specific activity: 49.1 KCi/mol) was obtained from NEN Research Products, DuPont Corp., Wilmington, DE.

Figure 1. The transfer of cortisol into the collector, and nonspecific binding (NSB) of cortisol to the collector membrane during 30 min of exposure to an artificial saliva medium.

Preparation of artificial saliva medium. We prepared a medium approximating the protein and electrolyte content of saliva: amylase 0.6 g/L; lysozyme 0.2 g/L; mucin 0.7 g/L; IgG 0.2 g/L; albumin 75 mg/L; inorganic phosphate 2.5 mmol/L; Na^+ 60 mmol/L; K^+ 18 mmol/L; urea 5 mmol/L; I^- 1.5 mmol/L; Cl^- 50 mmol/L; pH 7.8. As a bacteriostat, 0.2 g of thimerosal per liter of solution was added.

Transfer of cortisol. Five polypropylene bags containing artificial saliva were equilibrated overnight. This solution was discarded and the bags were filled with 50 ml of artificial saliva containing 1.5 ng/ml of cortisol and 127 pg/ml of ^3H -cortisol (23,000 cpm/ml). The temperature of the solution was maintained at 37°C (body temperature). Three ultrafiltrate devices were added to each bag, and the bags were gently massaged by hand in a water bath at 37°C for varying periods of time (5, 10, 15, 20, and 30 min). After each time period, one bag was opened and the cortisol in the ultrafiltrate and in the external solution was measured by means of β -radiation of ^3H -cortisol.

Nonspecific binding. To determine the extent of cortisol binding to the membranes of the device, the membranes used in this experiment were blotted on absorbent paper and immersed in 10 ml of scintillation cocktail. Cortisol taken up by the membrane was measured by means of β -radiation of ^3H -cortisol. Correction for quenching by the membrane material was not required. In prior experiments, no appreciable quenching of β -radiation from a known amount of ^3H -cortisol was found in the presence of the membrane of the device.

Results

The transfer of cortisol from the external medium into the collector increased from about 60% after 5 min to about 77% after 30 min (Figure 1). At approximately 8 min of incubation, there was about 65% transfer. The nonspecific binding of cortisol to the semipermeable membrane was approximately 0.3 fmol/mm² and changed little over time. Because the total surface area of the osmotic device is 1,900 mm², about 0.2 ng per device were nonspecifically bound.

STUDY 2

Accuracy of the Device in Humans

The second study was conducted in vivo to compare the accuracy of the saliva-filtering approach with whole saliva.

Method

Subjects. We collected samples from 5 healthy young adults (2 women, 3 men), who were habitual cigarette smokers (for at least 3 years) participating in a research study on abstinence from nicotine. The subjects were studied over 4 consecutive afternoons. They smoked as usual all day on Day 1 and then refrained from all nicotine on the subsequent 3 days.

Specimen collection. Whole-saliva and ultrafiltrate samples were collected from each subject during each of the first 3 days of the study; two pairs of samples were obtained on Day 4; thus, five sets of saliva specimens were accumulated for each subject, yielding 25 total sample pairs. For the collection, the device was moistened with tap water for 5 sec and placed in the subject's mouth. The subject moved the device around with his/her tongue and held excess saliva in the mouth to permit absorption into the vehicle. After 8 min, the subject expectorated excess whole saliva into a test tube, and also removed the device. The subject continued to spit into the tube until at least 2 ml of whole saliva was collected. Both the ultrafiltration collectors and test tubes containing whole saliva were cooled immediately. Within 30 min, the device was frozen to -80°C, and whole saliva in the tubes was centrifuged at 3,000 rpm for 15 min. At least 1 ml of supernatant from whole saliva was separated and frozen for analysis.

Immunoassay. Cortisol in whole saliva and ultrafiltrate was quantitated by solid-phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles) adapted for the measurement of cortisol in saliva according to the manufacturer's recommendations, with some modifications. The assay was performed in 12 × 75 mm silicon-coated tubes with a total volume of 1.6 ml and 200 μl of sample volume. The sample volume was increased if required. A ^{125}I -labeled cortisol derivative was used as tracer. From the logit-log expression of the dose-response curve, we measured ($M \pm SD$) at 80% tracer inhibition, $0.147 \pm 0.012 \mu\text{g/ml}$; at 50%, $0.75 \pm 0.05 \mu\text{g/ml}$; and at 20%, $3.8 \pm 0.2 \mu\text{g/ml}$.

Correction for partial molal volume. The ultrafiltrate of saliva contains sucrose that occupies a certain volume of pipetted ultrafiltrate. Therefore, the pipetted volume has to be corrected for the partial molal volume. The total water concentration in the ultrafiltrate, C_W (g/ml), is a function of the relative density, D . The water concentration and the density follow the equation $C_W = 2.7 - 1.7D$, from which the actual concentration, C_{ACT} , can be calculated from the concentration measured in the ultrafiltrate, C_{UF} , by the equation $C_{ACT} = C_{UF}/C_W$. By weighing 1 ml on an analytical balance, we determined the average density of saliva ultrafiltrate, yielding a mean density of 1.142 ± 0.032 ($n = 35$). Thus, the average correction used for concentration was $C_W = 0.76$.

Results

Whole-saliva and ultrafiltrate cortisol concentrations followed a linear regression with a correlation coefficient of $r = .91$ (Figure 2). Two of the cortisol pairs from 2 subjects were identical and relatively high (see Figure 2), thus potentially overestimating the correlation; yet the correlation was only slightly reduced ($r = .84$) when these pairs were excluded. When the correction for partial molal volume was applied to the ultrafiltrate concentrations, the cortisol concentration in the ultrafiltrate averaged 79% of that found in whole saliva.

STUDY 3

Ultrafiltrate Cortisol and Individual Differences

In this study, we evaluated whether subject differences in depression and cigarette use predicted ultrafiltrate cortisol levels. Both depression (Arana & Mossman, 1988;

Figure 2. Cortisol concentrations in the ultrafiltrate (UF) as a function of cortisol concentrations in whole saliva from 5 human subjects. The regression equation is $C_{UF} = 0.26 + 0.61C_{WHOLE}$; standard error of estimate: 0.19 ng/ml.

Kalin & Dawson, 1986) and nicotine (Pomerleau & Pomerleau, 1990b; Wilkins et al., 1982) are known to covary positively with cortisol.

Method

Subjects and Procedures. Twenty 23- to 36-year-old adult smokers (10 women) were recruited from the local community to participate in the research project on abstinence from nicotine described under Study 2 above. On the first afternoon, the subjects came to the laboratory, smoked a single cigarette, and completed a depression questionnaire. An ultrafiltrate of saliva was collected with the device, as described in Study 2, 45 min after the cigarette was smoked. The ultrafiltrate was stored and cortisol analyzed using the immunoassay and correction for partial molal volume as in Study 2.

Depression measure. Depression was assessed with the Center for Epidemiologic Studies–Depression Scale (CES–D; Radloff, 1977), an inventory of 20 symptoms, which are rated on a 4-point scale (0–3) for the frequency that each symptom was experienced during the previous week. Possible scores range from 0 to 60; higher scores reflect increased depression. The CES–D is more sensitive to current than to long-standing depression, and it emphasizes affective and cognitive rather than vegetative symptoms. The instrument has been shown to have very high internal consistency and relatively high correlations with alternative questionnaire and interview measures of depression.

Results

Depression and daily cigarette frequency correlated significantly with ultrafiltrate cortisol (cortisol in ng/ml, $M = 1.5$, $SD = 0.6$, range = 0.6–2.8). As hypothesized, greater depression (CES–D, $M = 11.7$, $SD = 9.1$, range = 0–31) correlated with higher cortisol levels ($r = .46$, $p = .04$). Additionally, greater daily cigarette frequency ($M = 23.5$, $SD = 7.1$, range = 8–40) correlated with higher cortisol ($r = .64$, $p = .002$). Depression and cigarette frequency were not significantly correlated ($r = .08$), indicating that they were relatively independent predictors of cortisol.

DISCUSSION

This paper introduces to behavioral research a technology that facilitates the assessment of cortisol. The use of saliva obviates difficulties associated with urine and blood-based cortisol assessment, and the ultrafiltration technology appears to improve ease of collection and handling as well as accuracy. Subjects in this and other studies have reported that use of the device is more acceptable than repeated expectoration, and an ample supply of saliva is stimulated quickly, thus preventing “dry mouth.” Furthermore, the collection procedures and duration are standardized and behaviorally passive, minimizing interruption of subjects’ ongoing behavior. The device yields an ultrafiltrate free from viscous polysaccharides and mucoproteins—substances that make laboratory processing of saliva more difficult. Ultrafiltrate technology also has the potential to reduce measurement error by excluding blood components, food residues, enzymes, oral microorganisms, and other large particles that can confound the assay via cortisol binding or metabolism. Although samples should be refrigerated, brief storage at room temperature does not jeopardize the integrity of the specimen. Also, as suggested by its ability to assess the presence and amount of drugs such as cotinine (nicotine metabolite), phenytoin (anticonvulsant drug), and cocaine (Schramm et al., 1991; Schramm, Craig, Smith, & Berger, 1993; Schramm, Pomerleau, Pomerleau, & Grates, 1992), the device has potential clinical diagnostic value.

Cortisol collected using this technology appears to accurately reflect levels both in an artificial saliva medium and in whole human saliva. Approximately 75% of the cortisol in whole saliva transferred to the ultrafiltrate, and there was a very high correlation between cortisol concentrations in the ultrafiltrate and whole saliva. Utility of the device was demonstrated by replicating previous observations of individual differences in cortisol levels: subjects with greater depression scores and higher frequencies of daily cigarette use had higher levels of cortisol in the ultrafiltrate.

Clearly, further research on this technology is needed. The loss of 0.2 ng per device due to nonspecific membrane binding may result in some underestimation of cortisol at low concentrations. Yet this amount (0.2 ng) is minimal compared with a typical cortisol concentration of about 0.6–6 ng/ml in saliva, and considering that up to 10 ml of saliva flow past the device during the collection period. Nonetheless, it would be desirable to reduce the membrane binding, perhaps by using other membrane materials, such as a copolymer of polycarbonate and polyethylene (Gambro GmbH, Hechingen, Germany). The lower cortisol concentrations found in the ultrafiltrate than in whole saliva are probably due to hydrophilic/lipophilic interactions between cortisol and the membrane rather than to a prevention of cortisol

entry because of the pore size. A correction of saliva concentrations would be needed if direct comparisons with whole saliva or blood were desired. Although the collection of cortisol into the device changes minimally over 30 min in a stable, artificial saliva medium, it would be desirable to examine the rate of accumulation over the 8-min collection period used with humans. Although this technology appears to be appropriate for studies examining relatively stable or enduring individual or group differences in cortisol, further study is needed to confirm that the ultrafiltrate technology accurately assesses acute changes in cortisol as a function of physical, psychosocial, or pharmacological stimulation. It is expected that the device can assess short-term differences in cortisol levels, however, because it accurately reflects current whole-saliva cortisol concentrations, and whole saliva is sensitive to acute cortisol changes (Kirschbaum & Hellhammer, 1989).

Several other technologies have been developed to circumvent the collection and measurement problems associated with whole saliva. The use of a cotton plug ("Salivette") in the buccal cavity to accumulate whole saliva obviates some of the collection problems (Hellhammer, Kirschbaum, & Belkien, 1987). The large surface area binds some molecules, however, thereby limiting measurement accuracy, and the whole-saliva product still must be processed and is exposed to enzymes and other components that could increase measurement error (Schramm, Kidwell, et al., 1992; Vining & McGinley, 1986).

A promising filtrate technology similar to the device presented here has been developed and tested by Wade and colleagues (Shipley, Alessi, Wade, Haegele, & Helmbold, 1992; Wade, 1992a, 1992b; Wade & Haegele, 1991). The "oral diffusion sink" (ODS) is a small device, worn unobtrusively in the mouth, that accumulates substances by binding them to a specific antiserum in the device. A dialysis membrane prevents entry of larger components. Unlike the ultrafiltrate collector, the ODS is designed to accumulate substances over a period of hours, however, yielding an integrated value that is minimally affected by acute changes in cortisol levels. Wade and colleagues have demonstrated, in an elegant series of studies, the utility of the ODS for assessing cortisol and other steroids. Although both the ODS and the ultrafiltrate device filter out large substances and accumulate a medium suitable for the measurement of small molecules, the ultrafiltrate device is designed to measure the integrated concentration during brief collection periods (8 min). Although research has not directly compared these two approaches, both devices show potential for simplifying and improving saliva sampling of cortisol along with other molecules that may be of interest to behavioral scientists.

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