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Running Title: Salivary Annexin-A1

Abstract/Summary

Salivary cortisol is commonly used as a clinical biomarker of endocrine status and also as a marker of psychosocial stress. Annexin-A1 (AnxA1) is an anti-inflammatory protein whose expression is modulated by glucocorticoids. Our principal objectives were to (i) detect the presence of and (ii) measure AnxA1 protein in whole human saliva and to (iii) investigate whether salivary cortisol and AnxA1 are correlated in healthy humans. A total of 37 healthy participants (male and female) were used in the study. Saliva was collected using salivette tubes. Salivary cortisol and AnxA1 protein were sampled at between 3 and 6 time points over 24 hours and measured for cortisol and AnxA1 protein using specific ELISA's. The presence of salivary AnxA1 protein was confirmed by western blotting.

AnxA1 protein is detectable in whole human saliva, as detected by western blot analysis and ELISA. A diurnal rhythm was evident in both salivary cortisol ($P < 0.01$) and AnxA1 ($P < 0.01$) and was defined as a significant difference in time 0 (waking) samples compared to 'bed' (23.00 hrs) samples. AnxA1 protein did not exhibit an awakening response ($P > 0.05$), whereas salivary cortisol was significantly elevated between time 0 and 30 minutes post waking ($P < 0.001$).

AnxA1 protein correlates positively with salivary cortisol, indicating that cortisol is most likely a regulator of AnxA1 in human saliva.

Keywords: salivary cortisol, Annexin-A1, salivary biomarkers, diurnal rhythm, awakening cortisol response.

Introduction

The HPA axis is activated by physiological as well as psychological stressors and is also imprinted with a diurnal rhythm, with peak cortisol levels in humans being measured at around 0700h falling to a nadir in the late evening. A cortisol diurnal rhythm also exists in human saliva, where only the unbound steroid is present. Cortisol from the peripheral circulation diffuses through the epithelial cells of the three major extrinsic salivary glands via a trans-cellular route and is secreted into the oral cavity in saliva. Salivary cortisol has proven to be a robust and convenient biomarker and is extensively used to investigate clinical and psychological disease (Kirschbaum and Hellhammer, 1994) and correlates positively with plasma cortisol concentrations (Kudielka et al, 2009). However, a physiological role for cortisol in the oral cavity has not been determined.

Annexin-A1 (AnxA1; formerly lipocortin) is a glucocorticoid-modulated protein, which is responsible in part for mediating anti-inflammatory actions of glucocorticoids on the innate immune system, as well as being involved in glucocorticoid negative feedback inhibition of ACTH in the pituitary gland (Hannon et al, 2003). AnxA1 is expressed in abundance in human leukocytes (e.g. neutrophils) (Spurr et al, 2011) and is also expressed in endothelial and fibroblast cells. Oral epithelial cells, including cells of the buccal mucosa, tongue, small salivary glands as well as the parotid and submandibular salivary glands in humans (Dreier et al, 1998) also express AnxA1. This protein is also present in human mucosal secretions such as bronchoalveolar lavage fluid (Smith et al 1990) and more recently been detected in human saliva (Lorenz et al, 2011; Jarai et al, 2012).

Given the well established regulatory control that systemic cortisol exerts over neutrophil derived AnxA1 protein expression (Hannon et al, 2003), and the presence of cortisol in human saliva, we hypothesise that salivary AnxA1 may be regulated by salivary cortisol.

Materials & Methods

AnxA1 mouse anti-human monoclonal antibody (clone 1B) used in the AnxA1 sandwich Elisa and SDS PAGE was generously provided by Dr Jeff Browning of Biogen Corp, Cambridge MA, USA.

Human Subjects

Saliva was collected from seven healthy subjects (Approval No WHRI07020C) at three to four hour intervals over 24 hours and measured for cortisol and AnxA1 protein by EIA and ELISA, respectively. Saliva was also collected from 29 healthy subjects (9 males and 20 females; University of Westminster ethical approval; App. No. 10/11/09G) at three different time points on the same day for 3 days at (i) time of waking (Time 0); (ii) 30 minutes after waking (30 mins); (iii) immediately before sleep (Bed). Written and verbal explanations of the study design were provided before written informed consent was obtained. Healthy males and females were used in each study with a mean age of 30. Exclusion criteria included i) any subjects receiving prescription or over the counter medication in the week leading up to and during the study ii) significant oral disease.

Collection of whole saliva

Whole saliva was collected using a plain Salivette tube (Sarstedt, Germany) following the instructions from the manufacturer. Specifically, participants were requested not to eat or drink (except water), brush or floss their teeth for 30 minutes before giving a saliva sample. Participants were further requested to rest for a period of 1 hour before giving all saliva samples. Following sample collection saliva was stored at -80ºC until the day of the assay.

Human Annexin-A1 ELISA Assay

Total (full length and cleaved forms) human AnxA1 protein levels were determined in human whole saliva using an in-house ELISA which has previously been described (Goulding et al, 1990; Yazid et al, 2009; McArthur et al, 2010). Briefly, capture antibody (clone 1b) was diluted to a final concentration of 10 μg/ml in bicarbonate buffer, pH 9.6 and was incubated overnight at 4ºC. Blocking was achieved using 1% solution of BSA in phosphate buffered saline (PBS) buffer for 1 hour at 37ºC. Human recombinant AnxA1 protein (Scientific Proteins, Witterswil, Switzerland) was used to produce a standard curve of between 1-1000 ng/ml. Saliva samples were diluted 1:1 (50μl each) in PBS containing 1% Tween-20. Samples and standards were assayed in duplicate on a 96 well high binding ELISA plate for 1 hour at 37ºC (Costar, USA). Anti-rabbit AnxA1 polyclonal antibody (1:1000, Cat no. 71-3400, Zymed, Invitrogen, UK) was used for detection (final concentration 1 μg/ml in 1% tween-20/PBS buffer pH 7.4) and incubated for 1 hour at 37ºC. Detection was achieved using an alkaline phosphatase conjugated goat anti-rabbit conjugated secondary antibody (1:1000, Cat no. A-8025, Sigma, UK) and incubated for 1 hour at 37ºC. Visualisation was achieved by then incubating with phosphatase substrate (1mg/ml; Cat no. N2640, Sigma, UK). The samples were then measured after 15 minutes on a plate reader at a wavelength of 405 nm and off-set at 540 nm. Conditioned media from primary human neutrophils was used as a positive control in this assay. The intra-assay coefficient of variation (CV) was calculated as 6.66 %. The ELISA described does not allow for differentiation of full length from the cleaved form of AnxA1. The current ELISA measures total AnxA1. Samples of whole saliva isolated from four human subjects at 0700h were stored for 0, one, four, 24, 72 and 168 hours at 4ºC, before being frozen at -80ºC for one week. All samples were then defrosted on ice and assayed for AnxA1 protein by ELISA.

(Insert Figure 1 around here)

Cortisol EIA Assay

Saliva samples were measured for cortisol using a commercially available, high sensitivity salivary cortisol enzyme immunoassay kit (Salimetrics, UK). All samples were assayed for cortisol according to the manufacturer's instructions without modification as described (http://www.salimetrics.com/).

Isolation of primary human leukocytes for Western Blotting positive control sample.

Experiments using healthy volunteers were approved by the local research ethics committee (P/00/029 ELCHA). Informed consent was provided according to the Declaration of Helsinki. Blood was collected into 3.2% sodium citrate and diluted 1:1 in RPMI 1640 (Sigma, UK) before separation through a double-density gradient. Neutrophil cells were then prepared for western blotting.

Western blot analysis

Neutrophils and saliva samples were were subjected to standard SDSPAGE (12%) and incubated with the mouse anti-human AnxA1 antibody [clone 1B; dilution, 1:1,000] as previously described (Brancaleone et al, 2011).

Anti-microbial studies

The effect of AnxA1 and its N terminal peptides was studied on the common oral anaerobic bacteria *Streptococcus mutans* and *Porphyromonas gingivalis* using the broth incubation (Aduse-Opoku et al, 1995) and disk diffusion method (Health protection Agency, 2006) (described further in supplementary information).

Data handling and statistics

Data are reported as mean ± standard error mean (SEM). Statistical differences were determined by one way ANOVA followed by Tukey's *post hoc* test using Prism software (Version 5.0)

Results

Salivary cortisol concentrations measured in whole human saliva at set times throughout the course of the same day, demonstrated a normal diurnal rhythm in our population (Figure 1A). A cortisol concentration of 0.774 ± 0.134 µg/dl was measured at 0700h which decreased significantly (**, $P < 0.01$) to 0.113 ± 0.0245 µg/dl by 2400h. Salivary AnxA1 protein also displayed a diurnal rhythm, which mirrored the trend of the salivary cortisol measurements. The mean 0700h AnxA1 sample measured 75.366 ± 16.909 ng/ml decreasing significantly (**, $P < 0.01$) to 17.134 ± 5.454 ng/ml at 2400h. Analysis of the AnxA1 and matching cortisol data from Fig. 1A & B suggest that a significant positive correlation exists between cortisol and AnxA1 protein ($r = 0.621$, $P < 0.0001$, Figure 1C). No gender specific differences were found in the salivary Anx-A1 concentrations. The presence of immunoreactive AnxA1 protein in whole human saliva was confirmed using Western blotting (Figure 1D, lane 2). The blot further reveals the dominant presence of the ~33kDa cleaved form of AnxA1 (lower band) compared to the full length ~37.5kDa full length protein (upper band).

Figure 2A demonstrates the presence of an awakening cortisol response in our subjects as confirmed by a significant increase in cortisol between the time of waking (labelled Time 0) and 30 minutes post waking (***, $P < 0.001$). A diurnal rhythm was also present in these samples defined by a significant reduction in cortisol in the late evening (labelled Bed) compared to Time 0 (**, $P < 0.01$). Although salivary AnxA1 protein failed to show a significant awakening response (Figure 2B), but did confirm the presence of a diurnal rhythm

in this larger cohort of subjects (Time 0 compared to Bed samples,***, $P < 0.001$, n=29 for all samples). To exclude that protein degradation occurred in the saliva samples, AnxA1 was compared to the same samples frozen at -80°C immediately following collection (n=4). AnxA1 protein stored at 4°C for up to 7 days revealed that there was no significant decrease in immunoreactive (Figure 2C).

There were no anti-microbial effects of either AnxA1 protein or its N-terminal peptides on the two common oral microbes *Streptococcus mutans* and *Porphyromonas gingivalis* tested in this study (see supplementary data).

(Insert Figure 2 around here)

Discussion

The current data demonstrate the presence of secreted AnxA1 protein in human whole saliva and further indicate that salivary AnxA1 protein is diurnally regulated. Furthermore, salivary AnxA1 protein and cortisol are positively correlated within the same individuals, indicating that salivary cortisol may be regulating the diurnal profile in salivary AnxA1 protein. This data suggests that, unlike cortisol, AnxA1 protein does not exhibit an awakening response, however it does follow a diurnal rhythm with levels significantly higher in the morning than in the evening. The reason for the lack of correlation between the awakening cortisol levels and the AnxA1 protein is unclear and may indicate a lack of sensitivity of AnxA1 protein expression/release to rapidly rising cortisol levels during the awakening cortisol response. Furthermore, AnxA1 is inducible by glucocorticoids in many systems, although the sensitivity and temporal profile of this relationship has not been fully explored. Based on the current data and literature, we propose that locally produced AnxA1 protein from the major salivary glands, as well as the oral mucosa, is responsible for the AnxA1 protein measured in

whole saliva samples. We further hypothesise that unbound cortisol from the systemic circulation, is responsible for the release of AnxA1 protein into saliva.

There are a number of possible physiological roles for AnxA1 protein in human saliva. The oral cavity represents an ideal environment for pathogen entry into human hosts. *In vitro* data suggests that AnxA1 protein confers protection to human cells from cytomegalovirus (CMV) entry (Derry et al, 2007), suggesting an anti-viral role for human salivary AnxA1 within the oral cavity. Other aspects of host defence were also explored in the current study, namely the direct anti-microbial actions of AnxA1 and its N-terminal truncated peptides. The N-terminus of AnxA1 is reported to contain cleavage sites for elastase (Smith et al 1990) and proteinase 3 enzymes which cleave at valine and alanine residues (Vong et al, 2007). The data indicates that the majority of the imunoreactive AnxA1 protein detected in human saliva samples is in the truncated form (~33kD). This result raises the possibility that anti-microbial peptides, which are commonly found in saliva, may be released during the proteolytic cleavage of full-length AnxA1 protein in saliva. We therefore synthesised synthetic peptides based on predicted sequences from these known cleavage sites and investigated direct anti-microbial actions of these peptides, as well as human recombinant AnxA1 protein and the AnxA1 mimetic peptide Ac 2-26 (See Table 1). In our experimental settings, we could not demonstrate direct anti-microbial effects of the peptides tested against two common oral anaerobic microbes *S. Mutans* and *P. Gingivalis*. There does remain the possibility that these peptide sequences may act against other oral microbes not tested during the current experiments or that these peptides may fulfil another role within the oral cavity.

Neutrophils are recognised as being the key phagocytic cell within the oral cavity with ~$1.7x10^8$ cells/cm^3 in the junctional epithelium alone, far higher than neutrophil levels in the systemic circulation (which fluctuate between$1x10^6$ and $4x10^6$ cells/cm^3)(Rashmi et al, 2006).

Individuals suffering from dysfunctional systemic neutrophil migratory activity exhibit symptoms of severe periodontitis leading to tooth and bone loss (Waldrop et al, 1987). This is a striking reminder of the essential role of neutrophils within the oral cavity.

Glucocorticoid regulated AnxA1 is known to reduce neutrophil emigration in models of vascular inflammation (Allcock et al, 2001). Therefore we postulated that within the oral cavity AnxA1 protein also under glucocorticoid control to modulate neurophil trafficking into the oral cavity. Additional anti-inflammatory actions of AnxA1, relevant to these settings, also include the masking of leukocyte recognition mechanisms, specifically bacterial lipid A present on Gram-negative bacteria (Eberhard and Vandenberg 1998) and lipoteichoic acid on Gram-positive bacteria (Gotoh et al, 2005). These data, combined with the wealth of literature supporting a role for systemic AnxA1 as a homeostatic and pre-resolving mediator may indicate a new and potentially important physiological role for AnxA1 in balancing host defence and homeostasis within the oral cavity. Furthermore, the data highlights the potential use of salivary cortisol in combination with annexin-A1 to determine subtle differences in responses to stress in normal subjects compared with patient groups. The data also highlights a potential new role for AnxA1 as a novel salivary biomarker for inflammatory disorders (and perhaps other pathologies) or as a predictive marker for therapy outcome. Furthermore, these new results indicate a more important physiological role for salivary cortisol beyond its current role as a convenient biomarker.

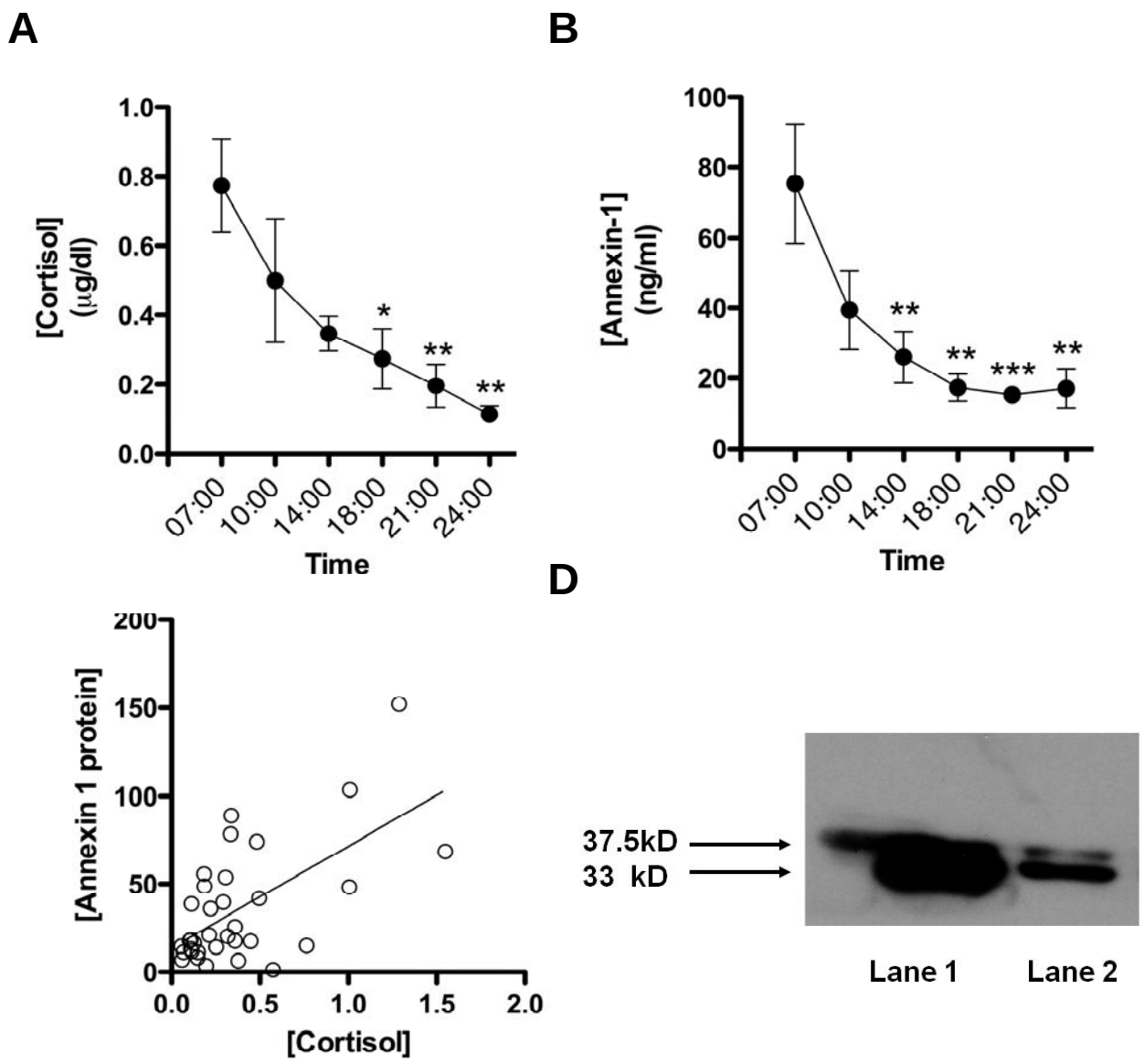

Fig. 1. Cortisol (A) and AnxA1 protein (B) measured by ELISA on samples of whole human saliva, collected from healthy human volunteers from 0700h until 24:00 hrs on the same day. Samples are expressed as ± SEM, n=7. Significance is indicated, *, $P < 0.05$, **, $P < 0.01$, *** $P < 0.001$ v's 0700h for each analyte. (C) Positive correlation between salivary cortisol concentration and salivary AnxA1 concentration measured in samples from A & B above ($r = 0.621$, $P < 0.0001$). The correlation of values was first tested to check if they were normally distributed using Mauchly's sphericity test followed by calculation of the Pearson coefficient using SPSS software (Version 15). **(**D**)** Detection of full length (37.5kD) & the N-terminal

cleaved form (33kD) of AnxA1 protein in the saliva (30µl) of a human subject (lane 2), measured by western blotting. Human neutrophil derived protein (25µg) was used as a positive control (lane 1). The intra-assay variation (CV%) was calculated as 6.66% for the AnxA1 ELISA. The intra-assay and inter-assay variations were calculated at 3.65% and 6.41%, respectively for the cortisol EIA.

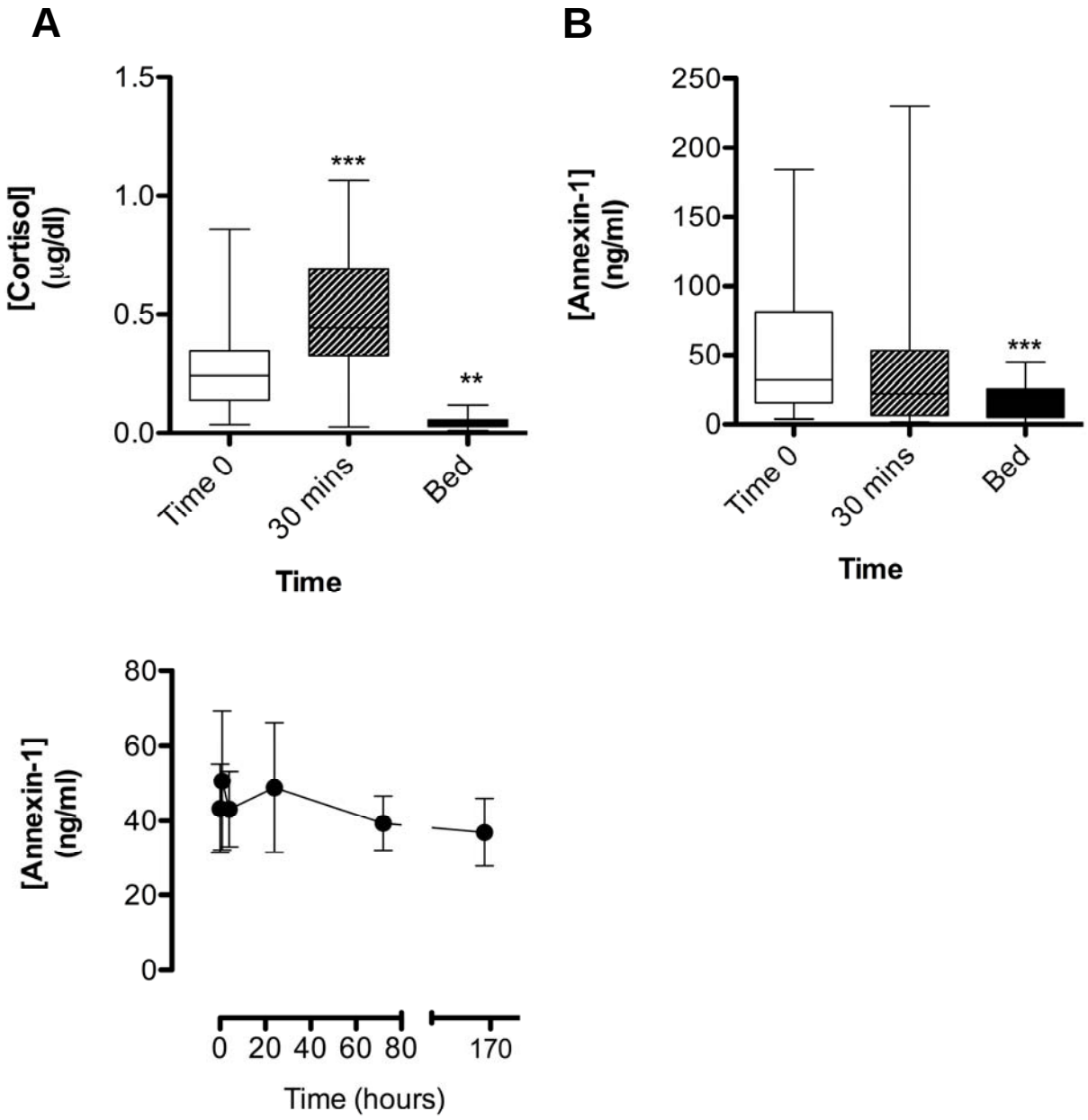

Fig.2. (A) Demonstration of a diurnal rhythm and awakening cortisol response in samples from 29 healthy human subjects. Awakening cortisol response was determined by

significance (***,$P < 0.001$) at 30 minutes post waking compared to the Time 0 samples. A cortisol diurnal rhythm was determined by comparing Time 0 to Bed samples (**,$P < 0.01$). (B) Diurnal rhythm in AnxA1 protein measurement was determined from Time 0 compared to Bed samples (***,$P < 0.01$). The lack of an awakening response was found in the salivary AnxA1 protein samples by comparing the mean 30 minute values to the Time 0 values ($P > 0.05$). All values are expressed as ± SEM, n=29. **(**D**)** Salivary AnxA1 stability experiment determined that there is no significant degradation of immuno-reactive AnxA1 protein in saliva samples stored at 4 °C for seven days. Samples represent whole saliva isolated from human subjects at 0700h and stored for 0, one, four, 24, 72 and 168 hours at 4ºC, before being frozen at -80ºC for one week. All samples were then defrosted on ice and assayed for AnxA1 protein by ELISA. Data is expressed as ± SEM, n=4, $P > 0.05$ at Time 0 compared to all other time points.

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