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Saliva chromogranin A in growing pigs: A study of circadian patterns during daytime and stability under different storage conditions

Damián Escribano, Ana M. Gutiérrez, María Fuentes-Rubio, José J. Cerón *

Interdisciplinary Laboratory of Clinical Analysis, Interlab-UMU, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100 Espinardo, Murcia, Spain

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ABSTRACT

Salivary chromogranin A (CgA) is considered to be a biomarker of activation of the sympatho-adrenomedullary system, and has recently been proposed as a useful indicator of the acute stress response in pigs. The aim of the present study was to determinate whether salivary CgA concentrations in healthy growing pigs exhibits any circadian pattern during the daytime, and to evaluate its stability under different storage conditions. A total of 80 pigs (40 in spring and another 40 in autumn) of two different ages and genders were used. To establish the circadian pattern, saliva samples were collected at 07.00, 11.00, 15.00 and 19.00 h on two consecutive days. Pooled samples were used for the stability study and were measured on the day of sampling and periodically for up to 360 days later. Samples were stored at 4 °C, −20 °C or −80 °C and the effect of repeated freezing and thawing was also evaluated.

No circadian pattern was detected for salivary CgA in either season and there were no significant effects of gender or age. However, mean salivary CgA concentrations were significantly higher ($P < 0.0001$) in the pigs sampled in autumn, compared to those sampled in the spring. Short term storage at 4 °C is recommended for up to 2 days, whereas frozen samples can be stored for 1 year at −20 °C or −80 °C, without substantial reduction in CgA values. In addition, samples can be frozen and thawed up to seven times without significant loss of the biomarker.

Introduction

Measurement in saliva of biomarkers such as chromogranin A (CgA) has the advantage of being non-invasive and is relatively stress-free when compared with blood sampling, which can be a confounding factor in stress models (Escribano et al., 2013). Furthermore, the technique is advantageous in studying the immediate response to a stressor, because multiple samples can be collected without greatly influencing the stress response (Heintz et al., 2011). One other advantage is that sampling can be carried out by individuals with a limited amount of training. For these reasons saliva is considered to be an ideal medium for evaluating stress responses in animals.

CgA is a 49 kDa acidic, soluble protein, which is stored and co-released with catecholamines from granules of the adrenal medullary and sympathetic nerve chromaffin cells (Blaschko et al., 1967; O'Connor, 1983). Although initially detected in chromaffin granules, this protein was later found to be widely distributed in the secretory vesicles of endocrine, neuroendocrine and neuronal cells (Winkler and Fischer-Colbrie, 1992; Hendy et al., 1995). CgA is secreted into the blood by the adrenal medulla and anterior pituitary gland during stress, with a smaller amount also released from sympathetic nerve endings (Taupenot et al., 2003). In humans (Taupenot et al., 2003) and dogs (Akiyoshi et al., 2005), CgA is considered to be an indicator of activation of the sympatho-adrenomedullary (SAM) system, being much more stable than catecholamines in the circulation.

CgA is also produced and released from the serous acinar and ductal cells of the human submandibular salivary gland (Saruta et al., 2005) and has been identified in salivary glands of other species, such as the rat and horse (Sato et al., 2002). It has been postulated that measurement of salivary CgA could be used as a sensitive and reliable quantitative tool for monitoring activity of the sympathetic nervous system, which constitutes the initial response to stress (Kanno et al., 1998; Nakane et al., 1998). Although the physiological role of CgA is still under investigation, salivary CgA might be a reliable and sensitive marker of SAM activation in humans (Gallina et al., 2011) and it has recently been used as a marker of the acute stress response in pigs (Escribano et al., 2013).

* Corresponding author. Tel.: +34 96 836 4722.
E-mail address: jjceron@um.es (J.J. Cerón).

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A variety of factors can influence the concentration of analytes in saliva, including circadian patterns of secretion, which need to be taken into account when saliva is used as a diagnostic sample (Lawrence, 2002). Circadian variation during the daytime can affect the concentration of salivary stress markers such as cortisol (Gallagher et al., 2002; Hillmann et al., 2008) or IgA (Muneta et al., 2010). In contrast, CgA concentrations in dog saliva have been shown to remain fairly stable (Kanai et al., 2008). However, the variation in salivary CgA is currently unknown in pigs.

The stability of a biomarker during sample storage is another important factor that needs to be considered. For logistical, financial and practical reasons, it is not always possible to analyse samples immediately after collection, and under these circumstances sample storage may be necessary (Ng et al., 2003). Stability studies have been undertaken for several biomarkers relating to the stress response, including cortisol (Garde and Hansen, 2005), IgA (Ng et al., 2003) and alpha-amylase (O'Donnell et al., 2009) in saliva, but investigations on the stability of CgA in saliva from veterinary species have not been performed.

The aims of the present study were to determinate whether salivary CgA secretion in healthy growing pigs exhibited any circadian pattern during the daytime in relation to season, gender or age, and to evaluate the stability of salivary CgA under different storage conditions.

Materials and methods

Saliva sampling and CgA analysis

All pigs included in the study were crossbred Duroc × (Landrace × Large White). Procedures involving animals were approved by the University of Murcia Ethics Committee and followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council of Europe, ETS Number 123).

Saliva was collected using Salivette tubes (Sarstedt) as reported previously (Gutiérrez et al., 2009). Pigs were allowed to chew a sponge attached to a flexible thin metal rod for 1 min. Each sponge was then placed in individual tubes and centrifuged at 3000 *g* for 10 min. Saliva samples were stored in Eppendorf tubes and frozen at −80 °C for the circadian variation experiment, or at 4 °C; −20 °C and −80 °C for the stability experiment.

CgA concentrations were measured in saliva using a validated time-resolved immunofluorometric assay (TR-IFMA) (Escribano et al., 2013) that performs with a high level of precision (intra- and inter-assay coefficients of variation of 6.23% and 5.82%, respectively), accuracy (coefficient, $r = 0.968$), and a lower limit of detection of 4.27 ng/mL. The calibration curve covered a range of salivary CgA concentrations from 46.8 to 1500 ng/mL. Saliva samples were diluted 1:4 with assay buffer, prior to quantification (DELFIA/Auto DELFIA wash concentrate, Perkin Elmer Life and Analytical Science). The enhanced fluorescence, proportional to the quantity of CgA in the sample, was measured in a VICTOR2 1420 multilabel counter (Perkin Elmer Life and Analytical Science). All analyses were performed in duplicate.

Evaluation of circadian pattern of salivary CgA secretion in pigs during the daytime

The experiment was conducted on a high sanitary/health-status farm in the southeast of Spain at the end October (the autumn season) and at the end of April (the spring season). A total of 80 animals (40 for each season) were randomly selected for inclusion in the experimental procedure. Pigs were housed in groups of 10: pens 1 and 2 housed 17- and 21-week old non-castrated males, respectively; pens 3 and 4 contained 17- and 21-week old females, respectively. Animals were kept under general commercial housing, with feeding and husbandry conditions conforming to European Union guidelines (Directive 2010/63/EU[1]), and had access to a nutritionally-balanced diet and water ad libitum.

Selected pigs were sampled on two consecutive days, to discriminate between cyclic and random sources of variation, at 07.00, 11.00, 15.00 and 19.00 h. This sampling regime had been previously used in pigs for the study of circadian rhythms of cortisol (Gallagher et al., 2002) and acute phase proteins (Gutiérrez et al., 2013). The temperature of the pens at these sampling time-points varied from 18 °C in the morning to 30 °C at midday in the month of April and from 15 °C in the morning to 25 °C at midday in the month of October.

[1] See: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF.

Assessment of the stability of salivary CgA

Samples were obtained from 35 randomly-selected pigs of different ages and gender, on the experimental farm of the University of Murcia. To have a sufficient volume of saliva for evaluation of CgA stability, the samples were combined into six pools of saliva, with three different CgA concentrations (two with high, two with intermediate, and two with low concentrations of CgA). The mean basal concentrations of CgA at the beginning of the experiment (day 0) were 1.27 μg/mL, 0.83 μg/mL and 0.21 μg/mL for the pools with high, intermediate and low concentrations, respectively. Aliquots of 100 μL were prepared from each pool to allow analysis after defined periods of storage at different temperatures. All aliquots were prepared in duplicate, with a protease-inhibitor cocktail (1 μL/mL, P8340; Sigma–Aldrich) added to one of the replicate samples. This cocktail was added before storage to prevent possible protein degradation by enzymes, as previously described by Hu et al. (2005). One aliquot from each pool was immediately analysed to determine a baseline (day 0) CgA concentration. The remaining aliquots were analysed after storage at 4 °C, −20 °C or −80 °C after 2, 5, 15, 30, 90, 180 and 360 days in the presence or absence of the protease inhibitor.

Two additional larger aliquots (1 mL) from each of the saliva pools were stored at −20 °C or −80 °C for evaluation of the effects of freezing and thawing on the CgA concentration. These aliquots were measured in parallel with the other aliquots, but were subjected to repeated freezing and thawing (seven cycles) at each of the eight analysis time points (Gutiérrez et al., 2011).

Statistical analysis

In both experiments, salivary CgA concentrations were evaluated for normality of distribution, using the Kolmogorov–Smirnov test for circadian patterns during the daytime, and the Shapiro–Wilk test for the stability study. As the results did not meet the normal distribution criteria, data were log transformed.

To study possible variation of salivary CgA due to a circadian pattern, a repeated-measures ANOVA test with a Bonferroni post hoc test were applied. The effect of seasonal variation on the circadian pattern was also studied using a two-way repeated measures ANOVA test and a Bonferroni post hoc test. Moreover, the overall mean concentrations of salivary CgA obtained in the two different sampling seasons were compared using an unpaired Student's *t* test. The mean concentration of CgA per pig was established as the mean of eight successive samplings.

To study the possible effect of gender and age on the circadian pattern, a two-way repeated measures ANOVA test and a Bonferroni post hoc test were performed for the 'sampling time-gender' and 'sampling time-age' influence independently. Differences in the mean concentrations of CgA in animals of different ages and also in animals of different sexes were studied using an unpaired Student's *t* test. The mean concentration of CgA per pig was established as the mean of eight successive samplings.

A repeated measures ANOVA and the Bonferroni post hoc test were used to evaluate changes in mean CgA concentration in saliva pools after storage at different temperatures (4 °C, −20 °C or −80 °C) and to assess the effects of repeated freezing and thawing. Changes that exceeded two intra-assay coefficient of variation (CV) of the CgA assay (6.23% × 2 = 12.46%) and had significant ($P < 0.05$) differences over time in relation to baseline concentrations (day 0 = 100% concentration) were considered to indicate a lack of acceptable stability for given storage conditions (Gröschl et al., 2001).

All statistical analyses were performed using a commercial statistics package (GraphPad Prism 5, GraphPad Software). A value of $P < 0.05$ was used to indicate significance in all analyses.

Results

Circadian pattern of CgA in porcine saliva during the daytime in two different seasons

No statistically significant differences were observed between sampling times over the two consecutive days, either in April or in October (Fig. 1). When the circadian pattern was compared in the two different seasons, no interaction was observed between the two factors (sampling time and season). However, salivary CgA concentrations were significantly higher in the group of pigs sampled in the autumn compared to those sampled in the spring ($P < 0.0001$).

Effect of gender and age on circadian pattern of CgA in porcine saliva during the daytime

There was no significant effect of gender on salivary CgA concentrations. Mean ± SD salivary CgA concentrations were