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Cholinesterase in porcine saliva: Analytical characterization and behavior after experimental stress

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ABSTRACT

The purpose of this study was to measure and characterize the enzyme cholinesterase (ChE) in porcine saliva, as well as to evaluate its behavior in experimental stressful conditions. The results of ChE characterization by using different substrates and the selective inhibitors ethopropazine and physostigmine showed that the main enzyme existing in porcine saliva was butyrylcholinesterase (BChE). An automated assay using butyrylthiocholine iodide as substrate was validated providing adequate reproducibility, linearity results and limit of detection. Salivary ChE was measured using the validated assay in two models of acute stress: twenty pigs stressed for 2 min with a nasal snare and other twenty pigs subjected to a short-term road transport. Salivary ChE significantly increased after restraint and transport stress in pigs, as well as the ChE to total protein ratio. In conclusion, BChE is the predominant isoenzyme in porcine saliva, it can be measured by the fast, simple and automated method described in this paper and it increases in the models of stress used in this study.

1. Introduction

Cholinesterases (ChE) are a group of enzymes capable of hydrolyzing choline esters. In mammals, two types of cholinesterases are present: acetylcholinesterase (AChE; Enzyme Commission number 3.1.1.7), also known as erythrocyte or true cholinesterase, which is mainly present in nervous cells and red blood cells; and butyrylcholinesterase (BChE; Enzyme Commission number 3.1.1.8), also known as plasma cholinesterase or pseudocholinesterase, which is found in plasma and several organs. In pigs, erythrocyte AChE hydrolyses acetylcholine and propionylcholine at similar rates, but not butyrylcholine; whereas plasma BChE hydrolyses the three substrates in a similar way (Tecles and Cerón, 2001).

The interest in ChE began as biomarkers of exposure to compounds that can inhibit this enzyme such as organophosphate insecticides (Gazzi et al., 2014). However, new roles have been proposed for these enzymes; for example, they are involved in neurotransmission and are related with neurological diseases such as Alzheimer's disease (Mushtaq et al., 2014) and attention deficit hyperactivity disorder (Archana et al., 2012). There are some evidences indicating a possible relationship between ChE and stress. Cholinesterase were related with anxiety in adults (Sklan et al., 2004) being the responses to anxiogenics modulated by cholinergic agents in mammals (File et al., 1998; Dazzi et al., 2001; Sanchez-Amate et al., 2001). In addition, increased ChE activity was reported in nervous tissue of mice suffering acute stress (Kaufer et al., 1998; Meshorer et al., 2002). Furthermore, acute stress elicits a transient increase acetylcholine (ACh) and a phase of enhanced neuronal excitability (Imperato et al., 1991), and ChE activity could be overexpressed in order to reduce the ACh excess (Meshorer et al., 2002). On the other hand, chronic stress has been reported to decrease serum BChE in rats (Tagliari et al., 2010).

Saliva is a biological fluid with increasing applications in veterinary medicine because it can be easily collected with minimum stress to the animals. Therefore, it can be considered as the ideal sample for analysis of stress biomarkers, especially in species where blood is difficult to obtain such as pigs. In this species various biomarkers of stress such as cortisol, chromogranin A (CgA) or alpha-amylase have been used in saliva (Fuentes et al., 2011; Escribano et al., 2013, 2015; Ott et al., 2014). Acetylcholinesterase is the main ChE in human saliva since its activity is inhibited by the AChE inhibitor neostigmine, but not by the BChE inhibitor ethopropazine (Sayer et al., 2004). However other reports indicated that BChE can be measured in saliva (Ryhänen et al., 1983; Finer et al., 2004; Archana et al., 2012). To the authors' knowledge, salivary ChE activity has not been characterized in porcine saliva, or evaluated as biomarker of stress in any species.

The hypotheses to be tested in this study are that: (1) ChE could exist and be detected in porcine saliva, and (2) ChE in saliva could change in its activity in stressful conditions. Therefore, the purpose of this study was to characterize ChE activity in porcine saliva, validate

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an automated spectrophotometric assay for its measurement and evaluate the possible changes in salivary ChE activity under two typical stressful situations commonly encountered in the swine industry: restraint by nose snaring or loop, which represents an acute stressor (Roozen et al., 1995; Geverink et al., 2002), and transportation of the animals since it has been also reported as a potent stressor (Sutherland et al., 2014). Additionally, the possible correlation between ChE results and CgA was studied. Chromogranin A is a biomarker of stress which indicates activation of the sympathetic system, being more sensitive than cortisol in some experimental stress conditions (Escribano et al., 2015).

2. Material and methods

2.1. Reagents and method for ChE measurement

Cholinesterase in saliva was analysed by an automated assay based on the Ellman method that was originally developed for whole blood (Tecles and Cerón, 2001), with some adaptations for saliva samples. A compact multiparametrical autoanalyzer for biochemical analysis (Olympus AU400, Olympus Diagnostica GmbH, Minneapolis, USA) was used. The temperature of analyses was 37 °C. Acetylthiocholine iodide (ATCI, Sigma-Aldrich Co, St Louis, USA), butyrylthiocholine iodide (BTCI, Sigma-Aldrich Co) and propionilthiocholine iodide (PTCI, Sigma-Aldrich Co) were used as substrates, each at a final concentration of 16.18 mM; 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Sigma-Aldrich Co) was used as chromophore (final concentration 0.29 mM). The rate of appearance of 5-thio-2-nitrobenzoic acid (reaction product between DTNB and thiocholine esters) per minute was detected at 405 nm wavelength. Cholinesterase activity was expressed as nmol of substrate hydrolysed/mL sample/min based on the absorptivity of the reaction product at 405 nm (13,600 M^{-1} cm^{-1}).

2.2. Method for chromogranin A measurement

Chromogranin A was measured in all the samples of the stress models in order to study the possible correlation of this marker with ChE. The concentration of salivary CgA was determined by time-resolved immunofluorometry assay (TR-IFMA), developed and validated in our laboratory as previously described (Escribano et al., 2013). The assay showed intra- and inter-assay coefficients of variation (CVs) below 10%, and lower limit of detection of 4.27 ng/mL.

2.3. Method for measurement of total protein (TP) concentration

Total protein concentration was measured in all samples in order to evaluate the influence of saliva concentration in ChE, by the calculation of the ChE/TP ratio. The normalization of analytes in saliva by dividing values per TP has been recommended by some authors to express results, in order to correct any influence on the results due to variations of the volume of saliva obtained per minute (Sayer et al., 2004; Oztürk et al., 2008; Bulgaroni et al., 2012). Total protein concentration in saliva was determined using a pyrogallol red colorimetric method (Protein in urine and CSF, Spinreact, Spain) at 598 nm employing an automated biochemical analyser (AU400, Olympus). Intra and inter-assay CVs were below 10% and the limit of detection was 0.005 mg/mL.

2.4. Animals

Saliva was collected from crossbred growing pigs [(*Sus scrofa domesticus*) Duroc × (Landrace × Large White)]. The animals were clinically normal without any sign of illness and they were vaccinated against *Mycoplasma hyopneumoniae* (Stellamune Mycoplasma, inactivated *Mycoplasma hyopneumoniae* NL 1042, Pfizer Animal Health, Madrid, Spain) and Aujeszky's disease virus (Porcilis Begonia, Intervet, Caracas, Venezuela) during the phases of lactation and fattening respectively.

All animals were in the last phase of fattening with 2–3 months of age at the Experimental Farm of the University of Murcia (Murcia, Spain). Pigs were given ad libitum access to a nutritionally balanced diet and water. The animals were housed in pens with a minimum space of 0.65 m^2 per animal (Council Directive 2001/88/CE of 23 October 2001 amending Directive 91/630/CEE concerning minimum standards for the protection of pigs). The temperature in the pens was kept at about 23 °C.

2.5. Sampling

Saliva was collected using Salivette tubes (Sarstedt, Aktiengesellschaft & Co. D-51588 Nümbrecht, Germany) containing a sponge instead of cotton swab (because these were less absorbent and released more saliva following centrifugation). The sampled pigs were allowed to chew the sponge, which was clipped to a flexible thin metal rod, until thoroughly moist. The sponges were then placed in test tubes and centrifuged at 3500 rpm for 10 min. Finally, saliva samples (approximately 0.5–1.0 mL per sponge) were stored in Eppendorf tubes and frozen at −80 °C until analysis.

2.6. Characterization of ChE activity in porcine saliva

For the salivary ChE activity characterization, porcine saliva samples were incubated with the selective inhibitors Ethopropazine (Etho, Ethopropazine hydrochloride, Sigma-Aldrich Co), a selective BChE inhibitor (Tasso et al., 2011), and physostigmine (Phy, Sigma-Aldrich Co), which selectively inhibits AChE (Zhan et al., 2010). Ethopropazine and Phy solutions were prepared in distilled water at 3 mM and 1 μM, respectively. Five saliva samples were obtained from healthy pigs and each sample was separated into 9 aliquots. Five aliquots were inhibited by adding Etho at increasing final concentrations (0.015, 0.05, 0.09, 0.15 and 0.5 mM), whereas other two aliquots were inhibited by adding Phy at different final concentrations (0.5 and 5 nM). The remaining two aliquots were incubated with an equal amount of diluent (distilled water). All aliquots were incubated at room temperature for 30 min. Then, ChE activity was measured as described above with the three substrates ATCI, BTCI and PTCI.

2.7. Analytical validation of the ChE assay

Two pools of saliva with different esterase activity were used for the analytical validation of the assay, which included:

- Reproducibility. Within- and between-run precision was calculated. To establish within-run precision, replicates of two pools of saliva samples were analysed six times in one day. Between-run precision was determined analysing the same pools on six different days within two weeks. Coefficients of variation (CV) were calculated as mean of the replicates divided by standard deviation times 100%.
- Linearity under dilution. Samples from each of the 2 pools of saliva were serially diluted with distilled water (75%, 50%, 25%, 12.5%) and assayed. The results were compared with those expected by linear regression analysis.
- Limit of detection. It is defined as the lowest concentration of an analyte that could be distinguished from a specimen with a value of zero, and it was calculated on the basis of data from 18 replicate determinations of the zero standard (distilled water), as the mean value plus 2 standard deviations.

2.8. Stress induction

A group of 40 healthy specific pathogen free pigs located at the Teaching Farm of the University of Murcia were used in the stress