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Short communication

Carbachol-induced *in vitro* secretion of certain human submandibular proteins investigated by mass-spectrometry

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ABSTRACT

Objective: To investigate protein content of saliva produced *in vitro* by samples of human submandibular gland following stimulation with the muscarinic agent carbachol.
Design: Tissue samples, obtained at surgery from seven patients and showing normal morphological appearance, were tested for 30 min: in absence of carbachol and atropine; in presence of carbachol (10 μM); in presence of carbachol (10 μM) and atropine (20 μM); or in presence of just atropine (20 μM). Medium was analysed by high-performance liquid chromatography–mass-spectrometry. Neither before nor during surgery were the patients exposed to drug treatments that were likely to influence the *in vitro* secretion.
Results: Proline-rich proteins (PRP)-1 and -3, peptide PC and PB, statherin, cystatins SN, S1 and S2 were invariably found in control gland tissue medium. Mean concentrations of these proteins/peptides in the medium were non-proportionally elevated following carbachol exposure to the gland tissues. Difference between basal release and carbachol-induced secretion achieved statistical significance as to all the proteins/peptides under study but for statherin. Atropine alone or atropine plus carbachol caused no significant changes compared to the basal release of proteins/peptides.
Conclusions: *In vitro* studies on salivary glands make it possible to study protein secretion from individual glands and thus, to reveal the contribution of the various types of gland to protein/peptide content of whole saliva. The disproportional responses to carbachol may imply that the proteins/peptides are not confined to the same cells or to the same intracellular locations and are therefore not secreted as packages at parasympathetic cholinergic activity.
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1. Introduction

The oral fluid is a mixture of saliva from major and minor salivary glands, fluid from the gingival pockets, cell debris and bacteria. Depending on type and intensity of the reflex stimulus different types of salivary glands are mobilised to various extent to create the most purposeful salivary response as to volume and composition under physiological condi-

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tions.[1] In animals, pure saliva is obtained for experimental purposes from duct-cannulated major salivary glands. In humans, studies are usually focused "on whole saliva", i.e. the oral fluid. Collection of saliva from a single gland is obtained by a device placed over the gland duct (or ducts).[2] However, usually no direct cannulation is performed in humans. Thus, it is difficult to avoid contamination of the saliva samples by the oral fluid. Whereas in animal studies, secretion is often evoked by stimulation of the parasympathetic and sympathetic innervations or by injecting selective receptor agonists into the blood stream, human secretion is usually evoked by taste or chewing, procedures most likely engaging a number of transmission mechanisms.[1,3]

In vitro observations allow the contribution of various salivary gland types to the composition of whole saliva to be studied. Furthermore, pieces of gland tissues can be exposed to selectively acting drugs, e.g. mimicking nerve-induced actions, without having to dissect nerves or to consider any systemic drug effects in the body. Whereas secretory *in vitro* studies on salivary gland tissues from animals are not uncommon, see e.g. Refs. [4–7], such studies on human salivary gland tissues are, on the whole, few.[8–11] Yet, the ultimate vindication for *in vitro* studies on salivary glands would be those using operational specimens from humans as pointed out by Garrett.[12] Hence, in the present study, the effect of the muscarinic receptor agonist carbachol on the *in vitro* secretion into the medium of certain proteins/peptides from pieces of human submandibular glands was analysed by applying high-performance liquid chromatography (HPLC) coupled to mass-spectrometry in order to investigate submandibular contribution to the protein content of mixed saliva in response to parasympathetic cholinergic activity.

2. Materials and methods

2.1. *Reagents and instruments*

All common chemicals and reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO, USA). The HPLC apparatus was a Thermo Finnigan (San Jose, CA, USA) Surveyor HPLC connected by a T-splitter to a diode-array UV–vis detector and to an LCQ Deca XP Plus mass-spectrometer. The mass-spectrometer was equipped with an electrospray ion (ESI) source. The reversed-phase chromatographic column was a Vydac (Hesperia, CA, USA) C_8 middle-bore column, with 5 μm particle diameter (column dimensions 150 mm × 2.1 mm).

2.2. *Collection and treatment of the glandular tissue*

Samples of human submandibular glands were obtained at tumour surgery from seven patients, six males and one female, aged 48–75 years. The glands had not been exposed to radiation. Furthermore, prior to surgery or during surgery the patients were not treated with drugs that were likely to interfere with the *in vitro* secretion under present experimental set-up. Informed consent was obtained in each case and permission was granted by the local ethical committee (A.S.L. 8, Cagliari). Immediately after the resection, gland tissue was washed twice with physiological saline solution and subdivided into pieces of about 5 mm long, 2 mm wide, 2 mm high. Incubations were performed as previously described.[13] Briefly, glandular tissue was incubated at 37 °C for 30 min in 10 mL of an oxygenated medium (6 mM Tris–HCl, 123 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$ and 5.5 mM glucose, pH 7.4) without or with carbachol chloride (10 μM), when appropriate, combined with atropine (20 μM) in 25 mL sealed Erlenmeyer flasks, placed in a water-bath and continuously shaken. At the end of incubation, the glandular tissue was removed from the medium, weighed and subjected to cytomorphological and ultrastructural analysis.[10] This examination of semithin tissue sections, allowed further assessment of the normality of the gland pieces incubated. Only incubation media from specimens showing a normal histological feature based on cytomorphological and ultrastructural analysis were used for biochemical characterization of their content. The mean wet weight of normally looking tissue, subdivided into small pieces, incubated in each Erlenmeyer flask was 53 ± 14 mg ($n = 22$). Medium was centrifuged ($6000 \times g$, for 10 min) to remove eventual gross material, lyophilized and stored at −80 °C, awaiting the following HPLC–MS analysis.

2.3. *RP-HPLC–ESI-MS analysis*

Lyophilized powder was dissolved in 150 μL of 0.2% aqueous trifluoroacetic acid (TFA), centrifuged at $10{,}000 \times g$ (10 min) and concentration of proteins in the supernatant determined by the bicinchoninic assay (Pierce, Rockford, IL, USA) corresponded to 0.15 ± 0.05 μg/(mL mg) of tissue under basal conditions and to 1.3 ± 0.3 μg/(mL mg) of tissue in stimulated experiments. 100 μL aliquots of the solution were directly injected into the HPLC–MS apparatus. The following solutions were utilized for the reversed-phase chromatography: (eluent A) 0.056% aqueous TFA and (eluent B) 0.050% TFA in acetonitrile–water 80/20 (v/v). The gradient applied was linear from 0 to 55% in 40 min, at a flow-rate of 0.30 mL/min. The T-splitter addressed a flow-rate of about 0.20 mL/min towards the diode-array detector and a flow-rate of about 0.10 mL/min towards the electrospray ionization source. The diode-array detector was set at a wavelength of 214 and 276 nm. The eluent was not directed towards the electrospray source for the first 5 min of separation, in order to avoid damage to the ion trap-mass-spectrometer (IT-MS), due to the elevated concentration of electrolytes (salts, small polar molecules). The ESI source spray voltage was 4.50 kV and the capillary temperature 220 °C with sheath gas flow-rate of 70 arbitrary unities. The ion trap apparatus operated in positive mode in the 300–2000 m/z range and mass spectra were collected every 3 ms in the positive ion mode.

2.4. *Characterization of peptides and quantification*

Proteins detected in incubation media corresponded in terms of both chromatographic elution time and ESI spectrum to salivary peptides characterized in human saliva in previous studies.[14–17] Molecular mass values were determined by deconvolution of ESI spectra, performed either by the software provided with the Deca-XP instrument (Bioworks Browser) or

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