EXAMINATION OF AMYLASE SECRETION IN RAT PAROTID GLAND

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INTRODUCTION

Salivary glands are typical exocrine glands and the function of the secreted saliva is promoting the digestive process and protecting the mucosa of oral cavity and oesophagus.

Salivary glands – depending on their secretory capacity – contribute to mass and composition of saliva transferring into the oral cavity differently. Thus the parotid is responsible for 80% of the amylase present in saliva.

α-amylase means the 30% of all parotid saliva proteins. It has been long known, that amylase, such as other digestive enzymes (lipase, tripsin) can be detected in blood serum. Their appearance in blood is generally believed to be the result of pathological events (for example inflammation) or accidental byproducts of exocrine secretion. But digestive enzymes are normal constituents of blood under physiological conditions as well, and a number of studies indicate that their levels in serum are regulated by physiological mechanisms. The two major sources of α-amylase in rat and man are the parotid gland and the pancreas. These sources are shown to contribute to serum amylase secretion equally in man whereas in rat, the pancreas seems to secrete much less amylase into the circulation than the parotid gland under physiological conditions. It has been also shown that pharmacological stimulation of parotid function by pilocarpine as well as parasympathetic nerve stimulation leads to an increase in serum amylase activity. In fasted rats food intake was also shown to elevate the level of parotid isoform of the enzyme. It is not known, however, how serum isoamylase level is affected by spontaneous feeding in nonfasted animals.

The principal control of salivary secretion is derived from autonomic nervous system through sympathetic and parasympathetic efferents. Other factors, however, such as mastication, nutrition and hormones may directly influence this process.

After stimulation impulses get through to the salivary glands supported by the autonomic nervous system and the secretion of saliva increases. Transmission of a neural (sympathetic - adrenerg and parasympathetic - cholinerg) signal to a salivary gland acinar cell occurs chemically via neurotransmitters, the first messengers of a secretory response. Neurotransmitters couple to specific cell surface receptor proteins resulting signal transduction mechanisms, which transfer the signal to the inside of the cell. There are two major transduction mechanisms operative in salivary gland acinar cells: the generation of cAMP (cyclic AMP) and the hydrolysis of PIP2 (phosphatidylinositol-4, 5-bisphosphate).
INTRODUCTION

Besides adrenerg and cholinergic regulations, non-adrenerg and non-cholinergic (NANC) mechanisms also can influence the functions of secretory cells. It is well known, that one of the main mediators of NANC nerves is the nitric oxide (NO), which is participated in the regulation of salivary secretion.

NO, one of the most smallest and simplest molecule (30 Da), is made by enzymes that are among the largest (=130-160 kDa) and most complicated. The reaction means the converting of L-arginine to products NO and L-citruline. The synthesis consists of two different steps and it requires a number of cofactors (NADPH, FAD, FMN, calmodulin, protoporphyrin IX (hem), tetrahydrobiopterin), and molecular oxygen.

NOS enzyme has three isoforms and they are the products of three distinct genes. Neuronal NOS (nNOS or NOS1) was discovered first in neurons. Expression of inducible form of NOS (iNOS or NOS2) occurs for various effects in many cells and tissues. Endothelial isoform (eNOS or NOS3) was located first in the vascular endothel. The isoforms nNOS and eNOS are designated after the first anatomical location, but their presence is proved in many other tissues also, for example in salivary glands.

NOS1 and NOS3 are constitutive enzymes (cNOS): they are expressed continuously in cells (in the cytosole or in the cell organelles), in contrast to the inducible isoform. They are dependent on Ca²⁺, because synthesis of NO starts when calmodulin binds Ca²⁺ and is linked to the cNOS. Stimuli that rise up the Ca²⁺-concentration in the cytosole can start the synthesis of NO. NO production proceeds till calmodulin dissociates from the enzyme.

In normal conditions, no measurable activity can be found for NOS2 (iNOS) in cells, but different stimuli such as bacterial toxins, infection and cytokines can cause the expression. In 2-4 h after the stimulus NOS2 can appear in macrophages as well as in many other cell types, like cells of the vascular endothelium and heart muscle, immune cells and in cells of the bowel. Its work is independent from the intracellular Ca²⁺ level as it binds calmodulin irreversible.

NO has many effects in the body, and also has a mediatory role in the gastrointestinal function. Lots of studies examined the location and distribution of the produced NO in the gastrointestinal tract, in different species, including human. NO-producing places (NOS enzyme) are located in salivary glands such as oesophagus, stomach, small and large intestines, rectum, pancreas, liver, gall bladder and bile duct. Widespread tissue distribution and species distribution is showed in NOS. There are many direct and indirect methods for measuring cellular NO production and NOS expression in acinar cell. Thus, NADPH-diaphorase histochemistry showed, that NO is widely distributed in the gastrointestinal tract, and in the salivary glands also.
Because of the small size and the lipophilic nature the endogen NO produced in the acinus cells of salivary glands can easily cross membranes by simple diffusion. However, it has a short half-life and breakdowns quickly so it possibly mediates signal interaction between adjacent cells. The main roles of NO are:

- ensuring adequate blood supply during long-term secretion
- retrograde messenger (negative or positive feedback mechanisms to the sympathetic and parasympathetic nerves)
- regulating cell growth and differentiation of surrounding nerve and vascular cells
- participating in the maintenance of the host defence barrier (against microorganisms as well as tumour cells)
- participating in pathogenesis of many diseases (for example Sjögren's syndrome, periodontal diseases, tumours)

The functions of saliva secreted by the salivary glands are complex. The main role of the saliva is to protect the oral cavity on the one hand by keeping the tissues moist and the other hand by secreting mucin (and prolin-rich proteins); they act as lubricants on the surface of the epithelium. Saliva also contains antibacterial substances, giving some protection against the bacteria, which inevitably invade to the body through the oral cavity.

Reduced salivary flow rate (sialosis) and/or changes in composition are affected in inflammation of salivary glands such as sialadenitis, Sjögren's syndrome and radiation damage. Reduced salivary secretion causes a dry mouth (xerostomia). The principal changes in ionic composition of saliva are increased concentrations of sodium and chloride and decreased concentrations of phosphate. There are changes in the relative concentrations of some minor salivary proteins. These changes may have some diagnostic value. As NO participates in the regulation of the amylase secretion, it can be useful therapeutically to understand what happens in salivary (for example parotid) glands under systemic inflammation in animals and what is the role of NO in it.

Lipopolysaccharides (LPS) are commonly used for modelling systemic inflammation; they can activate inflammation in tissues. LPS is consisted of carbohydrates and lipids, the name comes from here. LPS released from the outer membrane of Gram-negative bacteria is a major factor that contributes to the pathophysiology of bacterial infection. Endotoxin activates macrophages (monocytes) and granulocytes to release many inflammatory mediators such as various cytokines TNFα (tumor necrosis factor α), IL-1β (interleukin-1β), IL-6 (intelukin-6), IL-8 (interleukin-8)) and PAF (platelet activating factor). These products activate host defence systems that participate in eliminating the bacterial infection.
In experimental conditions, injection of LPS intravenously, can produce systemic inflammation in experimental animals after a few hours, as it was described previously. However, we investigated the effect of the LPS on the parotid gland firstly.
AIMS OF THE STUDY

The purpose of our study was twofold.

First we tried to investigate, whether the normal feeding influences the parotid isoamylase concentration in the serum in vivo. On the one hand, we examined the effect of 16 h fasting, 1 h feeding after fasting and 2 h feeding at night for the parotid tissue and serum amylase activities in rats. On the other hand, we evaluated how the different pharmacological and physiological stimuli (5 mg/kg pilocarpine or the same volume saline, sc.) can influence the amylase activities in the parotid and serum.

Second, we investigated the direct effect of NO on acinus cell in vitro. The presence of NOS enzymes was certified using many methods, and the role of NO in the physiological as well as pathophysiological processes of salivary (parotid) glands are supposed to be present. The data of the large number of in vivo experiments show the indirect effect of NO, acting through the circulation. In vitro investigations are much less, and they show, that NO has direct effect also on the parotid. The main purpose of the investigations described in this thesis was to examine the direct effect of nitric oxide in physiological and pathophysiological conditions. After intravenous administration of Escherichia coli endotoxin (LPS) we evaluated, how the systemic inflammation acts on the parotid amylase secretion.

We were mainly interested in:

1. In vivo investigation of the amylase secretion:
   1.1. Whether the normal feeding influences the parotid isoamylase level in the serum?
   1.2. How the amylase content changes in the parotid tissue and serum after pharmacological and physiological stimuli?

2. In vitro investigation of amylase secretion:
   2.1. How NO acts on basal and stimulated amylase secretion?
   2.2. How the LPS acts on the amylase secreting acinus cells, in direct or indirect manner?
   2.3. What is the role of the NO in the LPS-evoked inflammation of parotid gland?
METHODS

IN VIVO EXPERIMENTS

Female Wistar rats (250-320 g) were housed one by one in cages and under standard laboratory conditions in a 12:12 h light cycle.

1. Experimental design

In the first experiment, after 16 h fasting rats received either pilocarpine or saline (n=20, sc.). After 1 h, blood and parotid gland were collected.

In the second experiment 18 rats were used. After 16 h fasting half of the animals were fed with standard laboratory chow for 1 h, the other half received no food. After 1 h blood and parotid glands were collected.

In the third experiment 16 rats were allocated into 2 groups. All of the animals were fed ad libitum up to the start of the experiment. Then in the first just before the light was turned off at 19:00 h, food was withdrawn and animals were sacrificed 2 h later. Food was no withdrawn in the other group of rats; these rats were fed ad libitum and were also sacrificed at 21:00 h.

In all studies, upon completion of experiments, animals were sacrificed by thiopental-Na anaesthesia (ip., 50 mg/kg) followed by exsanguinations from the abdominal vein. Before this, blood was collected from the femoral artery.

2. Electrophoresis

After collection, blood was centrifuged at 2500 g at 4 ºC, and the serum was stored at -20 ºC until assayed. Electrophoresis of samples was performed in 0.9 % agarose gels at pH 8.6 in Veronal buffer at 80 V. Parotid and pancreatic amylase isoforms were separated utilizing their opposite charges at pH 8.6. Amylase activity was determined from isoenzyme bands of the gel.

3. Determination of amylase activity

For tissue analysis, following careful dissection, the parotid glands were trimmed out of fat, weighed and homogenized in Tris-buffer (pH=7.4, 10 mM). The homogenates were centrifuged and the supernatant was used for evaluation of amylase activity.

Amylase activity in tissue and serum was determined by starch-iodine colour reaction as described previously (Zelles et al, 1986). We used...
starch as substrate and the optical density of the reaction mixture was read at 620 nm. Dilution series of starch solution served as internal calibration points. The value of amylase activity was given as unit (U). One unit equalled 1 g starch digested in 1 min.

**IN VITRO EXPERIMENTS**

4. Inflammation producing in parotid gland

Male Wistar rats (200-250 g) were used. Escherichia coli LPS (0111:B4) was administered via tail vein (3 mg/kg, in saline) and glands were removed 5 h later. The LPS-induced oedema formation was evaluated by measuring the changes in the wet weight: dry weight ratio of the parotid gland.

For days prior to the study, the isoform non-selective NOS-inhibitor (N\(^6\)-nitro-L-arginine methyl ester, 0.1 mg/ml) was administered for a part of the animals.

5. Measurement of NOS activity

In the whole parotid tissue, Ca\(^2+\)-dependent (reflecting the constitutive NOS1 and NOS3 isoforms) and the Ca\(^2+\)-independent NOS activities (reflecting the inducible NOS2) were determined as the conversion of L-[\(^{14}\)C]-arginine to L-[\(^{14}\)C]-citrulline based on the method described previously (Salter et al. 1991).

After autopsy, tissues were homogenized in buffer containing EDTA (pH 7.4, 250 mg tissue/1 ml). Homogenates were centrifuged for 20 min (10000 g, 4 °C). Supernatants were mixed with Dowex resin and centrifuged for a further 10 min (10000 g, 4 °C). Sample supernatants were incubated in reaction buffer containing L-[\(^{14}\)C]-arginine. Besides this, saline, EGTA or N\(^6\)-nitro-L-arginine (L-NNA) was measured to every sample. The reaction was arrested by the addition (0.5 ml) of 1:1 v/v suspension of Dowex:water, which removed the excess L-arginine, as substrate. After addition of distilled water and settling for 30 min, the supernatant was removed for scintillation counting and the radioactivity of samples was measured. Protein content was estimated via spectrophotometric assay (Bio-Rad Protein Assay) and NOS activity was expressed as pmol/min/mg protein. Total NOS activity was defined as citrulline formation that was abolished by incubation in vitro with N\(^6\)-nitro-L-arginine (L-NNA, 1 mM). Basal L-NNA-sensitive activity that was abolished by EGTA was taken as calcium-dependent cNOS activity. In addition, calcium-independent NOS activity
(iNOS) was also determined as the difference between samples containing 1 mM EGTA and samples containing 1 mM L-NNA.

6. Isolation of parotid acinar cells

Isolated acini were prepared according to the procedure described previously by Telbisz and Kovács (1999) with minor modifications. Isolated acinar cells were placed into HEPES-buffer (pH 7.3) and incubated with different concentrations of the cholinergic agonist acetylcholine ($10^{-8}$-$10^{-4}$ mol) for 30 min at 37 °C. Thereafter, samples were centrifuged and the supernatant was removed. Finally, lysis buffer containing Triton-X was added to the acinar cell suspension and the cells dissolved.

7. Immunohistochemistry

From freshly isolated parotid acinar cells, a 1 ml suspension was prepared and centrifuged onto SuperFrost Plus microscope slides using a Shandon cytospin at 1500 rpm for 5 min. The cells were fixed in 4 % paraformaldehyde in phosphate-buffered saline (pH 7.4) for 30 min. Endogenous peroxidase activity was blocked with 0.3 % H$_2$O$_2$ in phosphate-buffered saline for 10 min, and after washing out traces of H$_2$O$_2$, non-specific binding sites were blocked with 7 % normal goat serum diluted in phosphate-buffered saline for 30 min. We used indirect immunoperoxidase or indirect immunofluorescent methods.

The cells were incubated overnight with 1:50 dilution of NOS2 monoclonal antibody. Following extensive washing steps with phosphate-buffered saline, cells were incubated with biotinylated anti-rabbit IgG for 2 h. Avidin-biotin complex reaction and 3'3'-diaminobenzidine (DAB) visualisation were performed according to the manufacturer's instruction.

Immunofluorescent staining was carried out on parallel slides. After fixation and washing steps, cells were incubated with the NOS2 antibody. After overnight incubation and further washing steps, cells were incubated with fluorescein-conjugated anti anti-rabbit IgG at 1:100 for 2 h.

Slides exposed to DAB-staining were counterstained with Papanicolau-A haematoxylin for 1 min, washed in tap water and mounted in Aquatex. Immunofluorescent staining was embedded in Biomeida Gel Mount. (Electron Microscopy Sciences, Washington, USA). Photographs were taken under bright field or fluorescence illumination (filter 488 nm) by using an Olympus microscope and digital camera with an original magnification of 20X.
8. Western blot analysis

The whole parotid tissue or the freshly isolated acinar cells were measured and homogenised on ice in TRIS-mannitol buffer (pH 7.4). Cellular debris was pelleted by centrifugation at 12000 rpm. Aliquots of 25 µl of total cellular protein were denatured. Equal amounts of protein samples were electrophoresed (100 V) in 7.5 % SDS-PAGE gel. After electrophoresis, the protein was electrophoretically transferred from the unstained gel to nitrocellulose membrane. The blots were probed with the primary mouse polyclonal antibody NOS2 at 1:2000 dilutions. The HRP-conjugated secondary antibody was used at 1:2000 dilutions also and the immunoreactive bands were visualized using ECL Advance Western Blotting Detection Kit.

9. Amylase secretion from isolated parotid acinar cells

The amylase content of samples was determined by using the method of Bernfeld (1965) with minor modifications. Starch suspension was used as the substrate of the amylase. The absorption of solutions was measured after using dinitro-salicylic acid by spectrophotometrically (546 nm). Dilution series of maltose solution was used as internal calibration points. The results were expressed as percent release of amylase, which was calculated as the ratio between amylase activity in the incubation medium (supernatant) and total amylase (supernatant+sediment).

STATISTICS

Data are expressed as mean ± S.E.M. and have been analysed by the analysis of variance (ANOVA), the Mann-Whitney non-parallel U-test and by the Tukey-Kramer multiple comparisons test.
RESULTS AND DISCUSSION

IN VIVO EXPERIMENTS

Electrophoretic mobilities of parotid amylase and serum parotid isoamylase were very similar, while the pancreatic amylase in serum samples moved to the opposite direction during electrophoresis. Our observations agree with previous studies, that parotid isoamylase is the primary component of circulating amylase.

Serum parotid isoamylase level in nonfasted rats was 5.78±0.79 U/l (n=10). In rats fasted for 16 h we found that this value was slightly decreased and reached the level of 4.10±0.68 U/l (n=10; p<0.01). Under experimental conditions parotid tissue amylase activity in nonfasted animals was 5.00±0.94 U/100 mg (n=10). Following 16 h food withdrawal this value was found to be 7.69±1.52 U/100 mg (n=10; p<0.01).

In the first experiment we studied the effect of pilocarpine, a parasympathetic agent on changes of serum and parotid tissue levels of the isoenzyme. We found a 39±9 % (n=20; p<0.01), decrease in tissue activity, and a 101±39 % (n=20; p<0.01) increase in serum activity after 1 h of pilocarpine administration. Our data are in agreement with previous studies.

In the next experiment, the effect of re-feeding was investigated in fasted animals. In rats fasted for 16 h, 1-h feeding resulted in a 35±15 % (n=18; p<0.01) decrease in tissue activity, and a 41±17 % increase in serum activity (n=18; p<0.05).

Finally, the effect of food intake on parotid and serum amylase was studied in nonfasted animals during the first 2 h of the dark period, that is, during the phase when rats consume a large amount of food following the light period when little food is taken. Under these experimental conditions, during the first 2 h of spontaneous food intake, tissue amylase level decreased by 27±7 % (n=16; p=0.01). In parallel, serum amylase level increased by 16±1 % (n=16; p<0.05).

Our study is the first to show that both parotid and serum amylase level is controlled by spontaneous food intake in rats. In ad libitum fed rats, serum amylase level increases and parotid amylase concentration decreases during the first hours of dark, the period when rats eat a major portion of daily food consumption. These antiparallel changes can be attributed to food-stimulated activation of neuronal and hormonal pathways leading to the discharge of parotid amylase into saliva, and also to the elevation of the enzyme activity in serum.
RESULTS AND DISCUSSION

IN VITRO EXPERIMENTS

1. Expression of NOS2 following LPS administration

Endotoxin (LPS) was administered as a bolus i.v. injection (3 mg/kg) 5 h before the experiment. The weight of animals, the wet weight of parotid glands and the amylase content did not change. However, the dry weight of parotid glands significantly changed and we determined the wet and dry weight ratio of the parotid gland as an index of inflammatory oedema, which elevated from 86,8±0,4% to 90,2±0,5% (n=10).

Using the citrulline-assay LPS significantly increased total NOS activity (from 1,38±0,08 to 1,77±0,10 pmol/min/mg protein, n=7, p<0,05) in the whole parotid tissue. This change reflected an increase in the Ca²⁺-independent NOS2 activity (0,022±0,008-rol 0,214±0,045-re, pmol/min/mg protein, n=7, p<0,05), since Ca²⁺-dependent NOS-activity did not significantly changed (1,36±0,11 and 1,53±0,05 pmol/min/mg protein before and after LPS challenge, respectively, n=7).

In addition, NOS2 activity was determined using by immunohistochemistry in isolated parotid acinar cells. LPS-treated acinar cells showed significantly stronger staining pattern as control cells.

Western blot analysis also demonstrated the expression of NOS2 protein in the tissue as well as in the isolated acinar cells of parotid gland after LPS administration.

Using the whole parotid tissue and isolated parotid acinar cells of the rat, our results agree with previous observations that LPS leads to NOS2 expression when determined after 5 h.

2. Parotid acinar amylase secretion following endotoxin challenge

Basal amylase secretion from the isolated parotid acinar cells was dose-dependently increased by the cumulative incubation with acetylcholine (10⁻⁸-10⁻⁴ mol) for 30 min. Systemic administration of LPS significantly reduced both basal and acetylcholine-evoked amylase secretion in the isolated acinar cells (by 55±10 % and by 49±6 %, at 10⁻⁶ mol acetylcholine, n=5-7, p<0,01). Treatment with the NOS-inhibitor L-NAME in vivo did not significantly affect either the subsequent basal or acetylcholine-stimulated amylase secretion, and did not influence the effect of LPS challenge.
Our results confirm that inhibition of amylase secretion occurs following systemic or local endotoxin challenge in the different salivary glands (parotid and submandibular gland), as well as in different species (rat, mouse). As these previous studies investigated whole glands or tissue slice, they could not clarify whether LPS impairs amylase release through indirect or direct pathways of the cellular level. Our present findings suggest that LPS reduces amylase secretion of parotid acinar cells by indirect mechanisms, since our studies on parotid acinar cells of rats showed that L-NAME in vivo did not affect either the subsequent basal or acetylcholine-stimulated amylase secretion with or without LPS administration.

In summary, endotoxin may attenuate oral barrier function against invading bacteria by decreasing parotid amylase secretion at its acinar cellular level. However, the present findings indicate that the basal, acetylcholine-evoked amylase release, or LPS-induced decrease in amylase release is not affected by the NOS-inhibitor, L-NAME. These findings thus suggest that under physiological and pathophysiological conditions, NO does not directly modulate basal or acetylcholine-evoked amylase secretion of the rat parotid acinar cell.
CONCLUSIONS

Our in vivo experiments demonstrated that spontaneous food intake could regulate the amylase content of parotid tissue and serum. Antiparallel changes in parotid amylase content and serum amylase activity for spontaneous food intake can refer to the endocrine function of parotid acinar cells.

Our in vitro experiments shows that NO does not have direct effect on the amylase secretion of isolated parotid acinar cells of rats neither in physiological, nor in pathological conditions. Bacterial endotoxin can reduce the amylase secretion of parotid acinar cells indirectly, and it can activate NOS2 enzyme both in tissue and in cellular levels. However, the excess NO, which is produced by activated NOS2 does not have effect on the amylase release of parotid acinar cells.
REFERENCES SERVING AS A BASIS OF THIS PH.D. THESIS


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