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Short communication

Endotoxin can decrease isolated rat parotid acinar cell amylase secretion in a nitric oxide-independent manner

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Abstract

Salivary mucus and amylase have an anti-bacterial nature. Bacterial endotoxin is considered to decrease mucus secreting cell activity by nitric oxide-dependent mechanisms. In this study, the actions of endotoxin on amylase secreting cell activity have been studied. Endotoxin (*Escherichia coli* lipopolysaccharide; 3 mg/kg, i.v., 5 h) evoked nitric oxide synthase 2 (NOS2) induction in the rat whole parotid tissue (assessed by Western blot and the citrulline assay) and in rat isolated parotid acinar cells (assessed by Western blot and immunohistochemistry), and reduced basal and acetylcholine-stimulated amylase secretion from these isolated cells. However, N^G-nitro-L-arginine methyl ester (0.1 mg/ml, 4 days in drinking water, yielding a dose of 25 mg/kg/day) did not affect amylase release under basal or acetylcholine-stimulated conditions, either in control acinar cells or those from endotoxin challenged rats. Thus, basal, acetylcholine-evoked or endotoxin-decreased cellular amylase secretion from rat isolated parotid acinar cells does not appear to be modulated by endogenous nitric oxide.

Keywords: Parotid acinar amylase secretion; Nitric oxide; Endotoxin; Salivary gland inflammation; NOS2 induction

1. Introduction

Saliva has important anti-bacterial properties and salivary glands secrete bacteria-binding proteins, including immunoglobulin A, mucin and amylase. Pro-inflammatory events during oral mucosal bacterial infection are reflected by a reduced saliva secretion, facilitating bacterial invasion into the body (Tenovuo, 2002). Incubation in vitro of mucus-secreting acinar cells with bacterial endotoxin lipopolysaccharide (LPS) reduces mucin secretion and leads to apoptosis, processes considered to be mediated by the expression of the inducible nitric oxide synthase (NOS) isoform, nitric oxide synthase 2 (NOS2) (Slomiany and Slomiany, 2002). LPS-triggered nitric oxide (NO) overproduction by NOS2 plays a crucial role in the inflammatory process of many tissues including the vasculature, gut and mucus-secreting salivary glands (Boughton-Smith et al., 1993; László et al., 1995; Kiss et al., 1997; László and Whittle, 1997; Lamarque et al., 2000; Slomiany and Slomiany, 2002).

To extend the knowledge of the actions of LPS on salivary secretion, particularly since the effects on amylase-secreting acinar cells are not known, the effects of challenge with LPS in vivo were studied on subsequently isolated preparations of rat parotid acinar cells and the possible involvement of NO was explored.

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2. Materials and methods

2.1. Endotoxin LPS challenge

Male Wistar rats (200–250 g) were used. The Ethical Committee of the Institute of Experimental Medicine approved the experiment.

Escherichia coli LPS (0111:B4; 3 mg/kg, i.v.) was administered as a bolus i.v. injection and glands were removed 5 h later. This dose and route of LPS, and the time of tissue removal following LPS were selected as optimum for the current study on the basis of previous studies with this particular LPS, where the time-dependent NOS2 induction in association with NOS2-dependent microvascular inflammation in a range of intestinal and extra-intestinal organs in the rat has been evaluated (Boughton-Smith et al., 1993; László et al., 1995).

For 4 days prior to the study, the isoform non-selective NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 0.1 mg/ml in drinking water, yielding 25 mg/kg/day) was administered. The dose, timing and route of L-NAME administration were taken from studies on the inhibition of NOS in gut inflammation (Kiss et al., 1997).

The LPS-induced oedema formation was evaluated by measuring the changes in the wet weight:dry weight ratio of the parotid gland.

2.2. Measurement of nitric oxide synthase enzyme activity

In the whole parotid tissue, Ca^{2+}-dependent (reflecting the constitutive NOS1 and NOS3 isoforms) and Ca^{2+}-independent NOS activities (reflecting the inducible NOS2 isoform) were determined as the conversion of L-[^{14}C]-arginine monohydrochloride to L-[^{14}C]-citrulline based on the method described previously (Salter et al., 1991; László et al., 1995; Kiss et al., 1997). After autopsy, tissues were weighed, dried and then homogenized (15 s, Ultra-Turrax homogenizer, 5 mm blade) in buffer (250 mg/ml, 4 °C) containing 10 mM HEPES, 32 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 2 μg/ml aprotinin at pH 7.4. Homogenates were centrifuged for 20 min (10,000×*g*, 4 °C). Supernatants were mixed with Dowex (AG 50W-8; 200–400, 8% cross-linked, Na^+ form) resin and centrifuged for a further 10 min (10,000×*g*, 4 °C). Sample supernatant (40 μl) was incubated for 10 min at 37 °C in reaction buffer comprising final concentrations of 50 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM valine, 1 mM dithiothreitol, 15.5 nM L-arginine, 1 mM L-citrulline, 0.3 mM NADPH, 3 μM flavin adenine dinucleotide (FAD), 3 μM flavin mononucleotide, 3 μM tetrahydrobiopterin and 0.17 μM of L-[^{14}C]-arginine. The reaction was arrested by the addition (0.5 ml) of a 1:1 v/v suspension of Dowex:water. After addition of distilled water and settling for 30 min, the supernatant was removed for scintillation counting. 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^G-nitro-L-arginine (1 mM). Basal N^G-nitro-L-arginine-sensitive activity that was abolished by EGTA, was taken as calcium-dependent constitutive NOS activity. In addition, calcium-independent NOS activity (NOS2) was also determined as the difference between samples containing 1 mM EGTA and samples containing 1 mM N^G-nitro-L-arginine.

2.3. Isolation of parotid acinar cells

Isolated acini were prepared according to the procedure described previously by Telbisz and Kovacs (1999) with minor modifications. Briefly, rats were anaesthetized with Nembutal (35 mg/kg, s.c.). The upper part of the body of rats was perfused in two steps (with Ca^{2+}-chelating buffer for 5 min and with Ca^{2+}-containing solution for 5 min). Thereafter, glands were removed and the tissue was digested in incubation medium containing 200 U/ml collagenase in a shaking water bath at 37 °C. After 30 min, the medium was replaced with fresh collagenase solution and the parotid was incubated for another 20 min. The tissue was dissociated using polyethylene pipettes decreasing in diameter, and filtered through nylon mesh (200 μm). After the purification of acinar cells through albumin gradient (4% solution of bovine serum albumin) with centrifugation (200×*g*, 2 min, 4 °C), cells were placed into HEPES solution (pH 7.3), and incubated with different drugs 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.

2.4. Immunohistochemistry

From freshly isolated parotid acinar cells, a 1 ml suspension was prepared and centrifuged onto SuperFrost Plus microscope slides (Menzel-Glasel, Germany) using a Shandon Cytospin at 1500 rpm for 5 min. The cells were fixed at room temperature in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 30 min. After three washing steps in phosphate-buffered saline, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in phosphate-buffered saline for 10 min, and after washing out traces of H_2O_2, non-specific binding sites were blocked with 7% normal goat serum diluted in phosphate-buffered saline for 30 min. The cells were incubated overnight at 4 °C with 1:50 dilution of NOS2 monoclonal antibody except the control cells, which were incubated with blocking medium. 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) visualization were performed according to the manufacturer's instruction (Vector Laboratories, Burlingame, CA, USA).

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-rabbit IgG at 1:100 for 2 h. All slides were washed in distilled water following a phosphate-buffered saline wash. 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 Biomedia 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 20×.

2.5. Western blot analysis

The whole parotid tissue or the freshly prepared acinar cells were homogenized (Ultra Turrax T8; 20000/min, 2×30s) on ice in TRIS–mannitol buffer (pH 7.4) containing 2.0 mM TRIS, 50.0 mM mannitol, 100.0 μM phenylmethylsulfonyl fluoride, 12.0 μM leupeptin and 0.5 mU/ml aprotinin, 0.5% Triton X-100. Cellular debris was pelleted by centrifugation at 12,000 rpm for 20 min at 4 °C. Aliquots of 25 μg of total cellular protein were denatured by mixing and boiling with 20.0 mM TRIS, 3.0 mM EDTA, 2% SDS, 10% mercaptoethanol, 20% glycerol and trace amount of Bromophenol blue. Equal amounts of protein samples were electrophoresed (100V) in 7.5% SDS-PAGE gel. After electrophoresis, the protein was electrophoretically transferred from the unstained gel to a nitrocellulose membrane (Amersham, Pharmacia Biotech., Buckinghamshire, UK). The blots were probed with the primary mouse polyclonal antibody NOS2 at 1:2000 dilution (Biotechnology Inc., Santa Cruz, USA). The HRP-conjugated secondary antibody was used at 1:2000 dilution (Biotechnology Inc., Santa Cruz, USA), and the immunoreactive bands were visualized using the ECL Advance Western Blotting Detection Kit (Amersham Biosciences).

2.6. Amylase secretion from isolated parotid acinar cells

Parotid acinar cells were isolated, and basal and acetylcholine (10^{-8}–10^{-4} mol)-induced amylase secretion was evaluated spectrophotometrically (Telbisz and Kovacs, 1999; Tsunoda et al., 2003). The amylase content of samples was determined by using the method of Bernfeld et al. (1965) with minor modifications, with starch suspension as the substrate of the amylase. The results were expressed as percent release of amylase, which was calculated as the ratio between amylase activity in the incubation medium and total amylase.

2.7. Chemicals

NOS2 monoclonal and polyclonal antibodies, Papanicolau-A haematoxylin stain and L-[U-^{14}C]-arginine monohydrochloride were obtained from BD Transduction Laboratories (USA), Reanal (Hungary) and Amersham (UK), respectively. All other compounds were from Sigma-Aldrich (Hungary).

2.8. Statistics

Data are expressed as mean±S.E.M. of n rats per experimental group, and have been analyzed by the Mann–Whitney non-parallel U-test or by the Tukey–Kramer multiple comparisons test, where appropriate. $P<0.05$ was taken as significant.

3. Results

3.1. Expression of parotid NOS2 and oedema following LPS challenge

In the whole parotid tissue, the total NOS activity was increased in the tissue taken 5 h after challenge of the rats in vivo with LPS compared with tissue from the control unchallenged rats (from 1.38±0.08 to 1.77±0.10 pmol/min/mg protein, $n=7$, $P<0.05$). This reflected an increase in the Ca^{2+}-independent NOS2 activity (upper left panel in Fig. 1), since Ca^{2+}-dependent NOS activity did not significantly change (1.36±0.11 and 1.53±0.05 pmol/min/mg protein before and after LPS challenge, respectively, $n=7$).

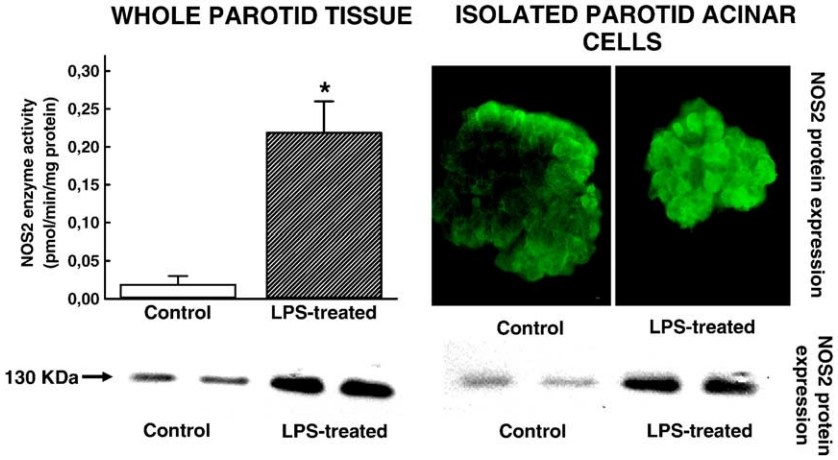

Fig. 1. The effects of in vivo challenge with endotoxin LPS on rat parotid tissue and isolated parotid acinar cells after 5 h. In the whole parotid tissue (left panels), LPS (from *E. coli*, 3 mg/kg, i.v.) induced an increase in Ca^{2+}-independent inducible nitric oxide synthase (NOS2) activity as assessed by the citrulline assay (expressed as pmol/min/mg protein; upper left panel) and NOS2 protein expression, as assessed by Western blot (lower left panel) 5 h after LPS administration. In the isolated rat parotid acinar cell (right panels), LPS-induced expression of NOS2 protein was assessed by fluorescent immunohistochemistry (upper right panel) and by Western blot (lower right panel). Data are expressed as mean±S.E.M. of $n=7$ rats per experimental group; *$P<0.05$ between control and LPS-treated groups.

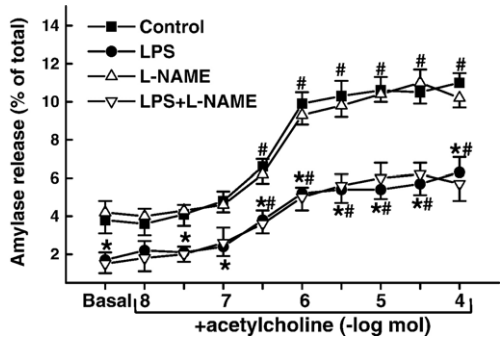

Fig. 2. Basal and acetylcholine-induced amylase release (expressed as a % amylase content of cell free and cell homogenate containing [total] incubation medium after a 30 min incubation) from rat isolated parotid acinar cells over a 5 h period (shown as solid squares). The effects of a challenge with endotoxin (LPS; from *E. coli*, 3 mg/kg, i.v. after 5 h) on basal and acetylcholine-induced amylase sections are shown as solid points. The actions of the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 0.1 mg/ml, in the drinking water, for 4 days previously) on parotid acinar cell amylase secretion in the presence (open down triangle) or absence (open up triangle) of LPS pre-treatment are also shown. Data are expressed as mean±S.E.M. of $n=5–7$ rats per experimental group. $^*P<0.05$ between control and LPS-treated groups. $^{\#}P<0.05$ between basal and acetylcholine-induced amylase secretion.

In addition, Western blot analysis on the tissue demonstrated NOS2 protein expression following LPS administration (lower left panel in Fig. 1).

In isolated parotid acinar cells, immunohistochemistry and Western blot analysis demonstrated the expression of NOS2 in the tissue 5 h following challenge with LPS, as shown in the upper and lower right panels in Fig. 1, respectively.

Administration of LPS in vivo increased the wet and dry weight ratio of the parotid gland as an index of inflammatory oedema from 86.8±0.4% to 90.2±0.5% ($n=10$, $P<0.001$).

3.2. 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), as shown in Fig. 2. Administration of LPS in vivo significantly reduced both basal amylase secretion and that provoked by the acetylcholine-induced amylase secretion (by 55±10% and by 49±6% [at the dose of 10^{-6} mol acetylcholine], respectively; $n=5–7$, $P<0.01$).

Treatment with L-NAME in vivo did not significantly affect either the subsequent basal or acetylcholine-stimulated amylase secretion in acinar cells from animals with or without LPS challenge (Fig. 2).

4. Discussion

Our present results in rat isolated parotid acinar cells confirm that inhibition of amylase secretion occurs following systemic or local endotoxin challenge in the different salivary tissues, the parotid and submandibular gland, as well as in different species, the rat and mouse (Rettori et al., 2000; Lomniczi et al., 2001; Espanol et al., 2003). Those previous studies investigated whole glands or tissue slices, and therefore they could not clarify whether LPS impairs amylase release through indirect or/and direct pathways at the cellular level. Our present findings suggest that LPS reduces amylase secretion of parotid acinar cells, at least in part, by direct mechanisms.

One of the most important mechanisms of LPS-induced inflammation is the overproduction of NO following the induction of the NOS2 isoenzyme. Intravenous injection of LPS leads to the time-dependent enhancement of NOS2 expression and inflammation in the gastrointestinal tract including the microvascular and epithelial cells (Boughton-Smith et al., 1993; László et al., 1994, 1995, Lamarque et al., 2000). The expression of NOS2 and the associated cell injury commence some 3 h after intravenous LPS challenge, and reach a plateau level by 5 h (Boughton-Smith et al., 1993; László et al., 1994). Using the whole parotid tissue and isolated parotid acinar cells of the rat, our results agree with these previous observations that LPS leads to inflammation and NOS2 expression when determined after 5 h.

Previous in vitro studies on mucus-secreting acinar cells indicate that LPS from *Porphyromonas gingivalis* leads to NOS2 expression and decreases the mucus secretory process (Slomiany and Slomiany, 2002). However, the present findings suggest that the role of LPS-induced NO induction on the impairment of mucus-secreting acinar cells and on amylase-secreting cells differ. Using mucus-secreting acinar cells from the rat sublingual gland, Slomiany and Slomiany (2002) demonstrated that incubation in vitro with L-NAME reversed the LPS-induced decrease in mucin synthesis, suggesting that LPS-induced NO production acted directly on the processes of mucus synthesis or release. In contrast, using isolated amylase-secreting parotid acinar cells of the rat, the current study has shown that the in vivo administration of L-NAME had no effect on the subsequent basal and acetylcholine-induced amylase secretion or its reduction by LPS challenge.

The mechanisms underlying these differences in the involvement of NO in LPS-induced inhibition of rat salivary mucus and amylase secretion are not yet understood, but may reflect differences between the endotoxin and the in vivo/in vitro protocols used for drug administration in the two studies, or to distinct cellular regulatory processes in the two secretory tissues involved. Other findings from a number of different preparations of salivary glands add to the complexity of the interpretation. Activation of PAR-2 leads to amylase and mucin secretion from the mouse salivary glands in vivo, actions mediated to some extent by NO-dependent mechanisms, although L-NAME only inhibited the stimulation of amylase secretion by a high dose of a PAR2 agonist, but not at lower agonist dose (Kawabata et al., 2000, 2002; Kawabata, 2003). Moreover, the salivation response to PAR-2 stimulation is decreased following LPS challenge and it was suggested that PAR-2, which has lower expression in the parotid than the sublingual gland, might be tonically activated and consequently desensitized during endotoxemia, but the processes and the involvement of NO are unclear (Kawabata et al., 2000, 2001; Kawabata, 2003). Other studies on salivary gland inflammation

using both in vivo systems and in vitro tissue slices have likewise suggested that endotoxin can inhibit salivary secretion through NO-independent (Rettori et al., 2000; Espanol et al., 2003) or only partially NO-dependent (Lomniczi et al., 2001) mechanisms. It was suggested that LPS causes the liberation of such cyclo-oxygenase-2 products as prostaglandin E2, which has key importance in the inhibition of salivary secretion (Lomniczi et al., 2001; Espanol et al., 2003). Thus, the differential role of NO and other products resulting from endotoxin challenge in the modulation of secretion from the different cell types of the salivary glands is not yet clear.

Previous studies have also suggested that under physiological circumstances, NO does not directly modulate cholinergic-stimulated amylase secretion from the isolated rabbit parotid acinar cells (Tsunoda et al., 2003), which is in agreement with our present results on NO-independent modulation of basal and acetylcholine-induced amylase release at the cellular level. In contrast, by using whole glands or tissue slices, inhibition of NO has been shown to decrease amylase secretion (Lohinai et al., 1999; Ishikawa et al., 2002). Thus, it is possible that NO acts indirectly on salivary amylase release in whole salivary gland tissue. NO produced in acinar cells and in surrounding structures might facilitate the actions or release of such neurotransmitters as noradrenaline or VIP that induce amylase secretion and which may interact in the regulation of secretion (Fujita-Yoshigaki et al., 1999; Roca et al., 2004), but such possibilities await exploration.

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 the LPS-induced decrease in amylase release is not affected by the NOS inhibitor, L-NAME. These findings thus suggest that under these physiological and pathophysiological conditions, NO does not directly modulate basal or acetylcholine-induced amylase secretion of the rat parotid acinar cell.

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