DETECTION AND ROLE OF THE FREE RADICAL NITRIC OXIDE IN THE EXOCRINE PANCREAS

Summary of PhD Thesis

ZSOLT EMBER MD

Tutor: Professor Erzsébet Fehér

Semmelweis University
Department of Anatomy, Histology and Embryology

Semmelweis University, Budapest
School of PhD Studies

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Summary

The free radical nitric oxide (NO) has several functions in various tissues. NO is well distributed in the gastrointestinal system including the pancreas and thought to be involved in the regulation of blood flow, motility and secretion. Although morphological reports provide evidence for the intrapancreatic production of NO, much less is known about the direct actions of NO exerted on the exocrine pancreatic tissues.

Neuronal nitric oxide synthase (nNOS) was both enzyme- and immunohistochemically localized in the porcine and rat pancreas. Reduced nicotinamide-adenine-dinucleotide-phosphate (NADPH) diaphorase (NADPHd) reactivity (R) and/or nNOS immunoreactivity (IR) was identified in the pancreatic ganglion cells and in nerve fibers next to acini and blood vessels. NADPHd R was also displayed by the porcine vascular endothelium, whereas the islet cells and the rat ductal epithelium occasionally showed nNOS IR. The distribution of nNOS in the neuronal and especially in the non-neuronal tissues showed age-related changes with increased contents of nNOS in the adult and ageing rat pancreas.

In order to demonstrate the direct actions of NO on *in vitro* rat preparations, we studied the effects of the NO donor sodium nitroprusside (SNP), the NOS substrate L-arginine (L-Arg), the NOS inhibitor N^G-nitro-L-arginine (LNNA) and the cyclic GMP (cGMP) analogue 8-bromo cGMP (8-Br cGMP) on basal and stimulated amylase secretion in isolated rat pancreatic segments. Stimulation was achieved by either acetylcholine (ACh) or electrical field stimulation (EFS). The changes in intracellullar free calcium concentration ($[Ca^{++}]_i$) were also investigated in isolated rat acinar cells. Both amylase output and $[Ca^{++}]_i$ were measured by fluorimetric methods. Our results show that both EFS and ACh resulted in marked increases in amylase output from pancreatic segments. SNP significantly reduced basal amylase output. This inhibitory effect could be mimicked by 8-Br cGMP. Combining SNP or 8-Br cGMP with EFS caused a significant decrease in amylase secretion compared with the response with EFS alone. This inhibitory effect was not evident when extracellular Ca^{++} concentration was elevated from 2.56 to 5 mM/l. SNP or 8-Br cGMP seemed to have no significant inhibitory effect on ACh-evoked amylase output compared with ACh alone. L-Arg or LNNA had no significant effects on either basal or EFS- or ACh-evoked amylase output from pancreatic segments. In isolated acinar cells, ACh induced the typical large

increase in $[Ca^{++}]_i$ whereas SNP and 8-Br cGMP significantly decreased basal $[Ca^{++}]_i$. Neither SNP nor 8-Br cGMP elicited any modification in ACh-evoked rise in $[Ca^{++}]_i$.

The results above indicate the presence of NO-containing (nitrergic) pancreatic nerves and the existence of a direct action of exogenous NO on rat acinar cells and also suggest its neuromodulatory role in the regulation of exocrine pancreatic secretion, possibly through the inhibition of ACh release.

Introduction

NO is a small free radical molecule with several functions in various tissues Produced by the isoforms of NOS enzyme, NO is supposed to be involved in both normal and pathophysiological processes of the entire organism in a concentration-dependent manner. Via the regulation of guanylyl cyclase activity the NO causes the relaxation of blood vessels and the inhibition of platelet aggregation, controls neurotransmitter release, acts as a neurotransmitter and also generated by immunological stimuli. NO is well distributed in the gastrointestinal tract and involved in the regulation of gastrointestinal motility and secretion.

Exocrine pancreatic secretion is controlled for the most part by the autonomic neurons and by enteroendocrine cells. In addition to the classic neurotransmitters and various neuropeptides that are apparently produced and stored in the pancreatic nerve cells, there is morphological evidence that nitrergic nerves are also present in the pancreas. NOS-containing perikarya and fibers have been shown in the pancreata of several species using enzyme- and immunohistochemical methods. However, such studies of the porcine pancreas are hardly found in the literature.

There are only a few data available on the correlation between pancreatic NO production and age. Though some results indicate a marked increase of NADPHd-containing rat intrapancreatic nerve cells during the first 3 months after birth, no data were found on the age-related distribution of NOS IR in pancreatic neurons, nerve fibers and parenchymal cells.

In contrast to the relatively high number of morphological studies on NO and the pancreas, few studies have focused on a physiological role of NO in the regulation of

pancreatic exocrine secretion. NO donors and NOS inhibitors have been demonstrated to modify pancreatic blood flow, pancreatic juice and enzyme secretion. The secretory effects of the above compounds in the *in vivo* studies are usually explained by the altered pancreatic circulation. More recently, the employment of either NOS inhibitors or NO donors in *in vitro* preparations has provided evidence that NO can exert a direct action on the pancreas independently on its vascular effects. Most research in this area seems to indicate that modulation of exocrine pancreatic function by NO is exerted via activation of guanylyl cyclase and generation of cGMP, although other pathways cannot be exluded. The interpretation of the results available to date is further complicated by the wide distribution of NO producing cells, the diversity of cellular targets and potential intracellular mechanisms of action and, finally, by the use of very different experimental approaches.

Aims

- Light microscopical enzyme- and immunohistochemical detection and localization of nNOS in porcine and rat pancreatic tissues.

- Investigation of age-related intrapancreatic distribution of nNOS immunoreactivity in four age groups of rats.

- Investigation of effects of the NO donor sodium nitroprusside (SNP), the NOS substrate L-arginine (L-Arg), the NOS inhibitor N^G-nitro-L-arginine (LNNA) and the cyclic GMP (cGMP) analogue 8-bromo cGMP (8-Br cGMP) on basal and stimulated amylase secretion in isolated rat pancreatic segments.

- Investigation of effects of SNP and 8-Br cGMP on intracellullar free calcium concentration ($[Ca^{++}]_i$) in isolated rat acinar cells.

Materials and methods

NADPHd enzymehistochemistry

Pancreatic glands of three swines weighing 150 kgs each were taken from a local slaughterhouse immediately after killing, rinsed in 0.1 M phosphate buffer (PB, pH 7.4), cut into small pieces then immersion-fixed in a solution containing 4% paraformaldehyde in 0.1 M PB for one week. After fixation the tissue was sectioned into 20-25 μm-thick slices using a Vibroslice equipment. After washing in 0.01 M phosphate buffered saline (PBS) the sections were incubated for 1.5 hrs in the dark at 37°C in 0.1 M PB containing 1.5 mg ml^{-1} NADPH, 0.75 mg ml^{-1} nitro blue tetrazolium and 30 μl ml^{-1} Triton X-100. In order to terminate the reaction, the sections were rinsed in cold 0.1 M PB. For the control specimens where NADPH was omitted, no NADPHd reactivity occurred.

NOS immunohistochemistry

5-day old neonatal (n=8, average weight 10 g) Wistar rats of either sex were anaesthetized with ether. 2-month old young (n=8, average weight 100 g), 4-month old adult (n=8, average weight 200 g) and 15-month old aged (n=4, average weight 300 g) Wistar rats of either sex were anaesthetized with intraperitoneal injections of ketamine and perfused transcardially with 4% paraformaldehyde in 0.1 M PB (pH 7.4). Pancreatic glands of the four groups were removed, cut into smaller pieces and placed in the same fixative solution for two weeks. The specimens then were processed for light microscopical immunohistochemistry on dewaxed 12 μm thick sections. Pancreatic glands of three swines were taken from a local slaughterhouse immediately after killing, rinsed in 0.1 M PB cut into small pieces then immersion-fixed in a solution containing 4% paraformaldehyde in 0.1 % PB for two weeks. After fixation the tissue was sectioned into 20 μm-thick slices. After washing in 0.01 M PBS the sections of rat and porcine pancreas were incubated for 24 h at 4 °C with rabbit polyclonal anti nNOS serum (dilution 1:1000). Following rinses in PBS the sections were incubated for 1 h at room temperature in biotinylated donkey anti rabbit secondary serum (dilution 1:70) and then in streptavidin biotinylated horseradish peroxidase complex (dilution 1:50). Sites of immunoreaction were visualized by incubating the specimens in 5 mg diaminobenzidine in 10 ml tris (hydroxymethyl) aminomethane (TRIS) buffer containing 120 μl 0.3% H_2O_2. For the control specimens where either the primary antiserum was omitted or replaced by normal

rabbit serum or the biotinylated secondary serum was replaced by normal donkey serum during processing no immunostaining occured.

Measurement of amylase output

4-month old Sprague-Dawley rats of either sex (n=20, average weight 200 g) were sacrified by a blow to the head and cervical dislocation. Pancreata were rapidly removed, cut into small segments (100-150 mg) and placed in modified Krebs-Henseleit (KH) solution (mM: NaCl, 103; KCl, 4.7; CaCl, 2.56; MgCl, 1.1; $NaHCO_3$, 25; NaH_2PO_4, 1.15; D-glucose, 2.8; sodium pyruvate, 4.9; sodium fumarate, 2.7 and sodium glutamate 4.9). KH solution was kept at pH 7.4 and 37 °C while being continously gassed with 95%O_2 / 5% CO_2. Pancreatic tissue segments were placed in a Perspex flow chamber and superfused with KH solution at 37 °C. Amylase concentration in the effluent from the chamber was measured using an on-line fluorimetric method. Fluorescence, a linear function of amylase concentration was monitored on a chart recorder. Basal, electrically stimulated and secretagogue-evoked amylase outputs were all expressed as units (U) ml^{-1} (100 mg tissue)$^{-1}$. Electrical field stimulation (EFS; parameters: 50 V, 20 Hz and 1 msec pulse width) was achieved by two silver electrodes embedded in the flow chamber. Known concentrations of either ACh (10^{-5} M), SNP (10^{-3} M), L-Arg (10^{-3} M), LNNA (10^{-3} M) or 8-Br cGMP (10^{-5} M) were directly added to the superfusing KH solution. The effects of EFS were examined in combination with either SNP, L-Arg, LNNA or with 8-Br cGMP in normal (2.56 mM) and with SNP in low (1.25 mM) and high (5 mM) calcium medium, respectively. Moreover, ACh was applied in combination with either SNP, LNNA or with 8-Br cGMP in normal calcium medium.

Measurement of intracellular calcium concentration

2-month old female Sprague-Dawley rats (n=8, average weight 150 g) were sacrified by a blow to the head and cervical dislocation. Pancreata were removed, finely cut and placed in modified Krebs-Ringer-HEPES (KRH) solution (pH 7.4) composed of (mM): NaCl, 130; KCl, 5; N-2-Hydroxyethilpiperazine-N'-2-ethanosulphonic acid (HEPES), 20; KH_2PO_4, 1.2; $MgSO_4$, 2.0; D-glucose, 10; $CaCl_2$, 1.0; trypsin inhibitor, 1.0 mg ml^{-1} and bovine serum albumin (BSA), 0.2% w/v. The tissue was then digested in two stages into acinar cells with

collagenase. Acinar cells were loaded with 2 μM fura-2 acetoxymethyl ester (fura-2 AM) for 40 min at 20 °C and washed in BSA deficient KRH solution. Aliquots of acinar cell suspension were placed in quartz cuvettes in an LS-50 spectrofluorimeter. Cells were continously stirred, maintained at 37 °C and scanned at excitation wavelenghts of 300-400 nm and the fluorescence was measured at 510 nm. Either ACh, SNP, 8-Br cGMP, SNP in combination with ACh or 8-Br cGMP in combination with ACh in known concentrations was added directly to the cell suspension. At the end of each sample the maximum and minimum fluorescence values were measured following the addition of 5 μM digitonin and 10 nM ethylene (bis)oxonitrilo tetraacetic acid (EGTA), respectively. The final intracellular free calcium concentration ($[Ca^{++}]_i$) was calculated by a previously described method.

Statistical analysis

All data provided were expressed as means ± standard error of the mean (S.E.M.). Data were compared using Student's *t*-test and only values with $p < 0.05$ were accepted as significant.

Results

NADPHd enzymhistochemistry and nNOS immunohistochemistry

Porcine pancreas NADPHd reactivities or nNOS immunoreactivities were observed in the majority of porcine pancreatic ganglion cells where stainings were mainly restricted to the cell bodies but less frequently appeared in the initial segments of neural processes, as well. However, some neurons that remained unstained were present in all the pancreatic ganglia examined. NADPHd or nNOS positive thin nerve fibers were seen within the nerve bundles of interlobular spaces and around blood vessels. The NADPHd reactive fibers where less numerous and lightly stained comparing to the higher density and more intense staining of nNOS immunoreactive fibers. Strong NADPHd positivity but no nNOS immunoreactivity was displayed by the vascular endothelium where the reaction product was seen in the

peripheral regions of the cytoplasm of endothelial cells, giving a network-like pattern to the tissue. Most of the islet cells showed strong nNOS immunoreactivity. However, NADPHd reactivity was not detected in the porcine pancreatic islets. Ductal epithelial cells and acinar cells seemed to be NADPHd / nNOS negative.

Rat pancreas nNOS immunoreactive perikarya of pancreatic ganglion cells were observed in the interlobular spaces in all the four age groups. Immunoreactive ganglion cells were more frequently found in the neonatal and young groups. Immunoreactive thin nerve fibers were regularly seen in the vicinity of acinar cells and pancreatic blood vessels of rats at all ages. The epithelial cells of intercalated ducts of adult rats showed strong NOS immunoreactivity while less intensive staining occured in the aging ductal epithelium. Weak positive staining appeared in most of the adult islet cells. The majority of peripheral islet cells of aging rats also became labelled.

Measurement of amylase output

The mean (± S.E.M.) basal amylase secretion was 6.94 ± 0.4 U ml^{-1} (100 mg tissue)$^{-1}$. Both EFS and ACh resulted in marked increases in amylase output from pancreatic segments. SNP significantly reduced basal amylase output. This inhibitory effect could be mimicked by 8-Br-cGMP. In low and normal Ca^{++} media, combination of SNP with EFS resulted in a significant decrease in amylase secretion compared with the response with EFS alone. In high Ca^{++} medium, SNP had no significant effect on the EFS-evoked amylase output. Combination of SNP with ACh had no significant effect on the amylase output compared with that of ACh alone. Combination of 8-Br cGMP with EFS resulted in a significant decrease in amylase output compared to the response with EFS alone. Combination of 8-Br cGMP with ACh had no significant effect on the amylase output compared with that of ACh alone. L-Arg or LNNA had no significant effects on either basal, EFS- or ACh-evoked amylase output from pancreatic segments.

Measurement of intracellular calcium concentration

The mean (± S.E.M.) basal $[Ca^{++}]_i$ in fura-2 AM loaded pancreatic acinar cells was 182 ± 7 nM /l. ACh induced a large increase in $[Ca^{++}]_i$ whereas both SNP and 8-Br cGMP was observed to have a significant inhibitory effect on $[Ca^{++}]_i$. When either SNP or 8-Br cGMP was combined with ACh, $[Ca^{++}]_i$ showed no significant changes compared with the response to ACh alone.

Discussion and conclusions

The enzyme- and immunohistochemical results of this study indicate the presence of nitrergic nerves in both the porcine and rat exocrine pancreas. nNOS immunoreactive fibers were regularly seen in the vicinity of pancreatic acini and blood vessels. These findings are in accordance with those of previous studies. The distribution of nNOS immunoreactivity showed age-related changes in the rat pancreas. The increased content of nNOS in adult and aging ductal epithelial cells and islet cells may suggest that the paracrine mediatory role of NO becomes more pronounced with age.

The presence and distribution of NOS-containing nerves may suggest a transmitter / modulatory role of NO in the exocrine pancreas. Previous studies have clearly shown the cholinergic nature of the majority of extrinsic and intrinsic nerves supplying the exocrine pancreas. In this study, the intrinsic nerves of rat pancreatic segments were electrically stimulated by EFS. Results of previous experiments show that EFS evokes marked increases in tritiated choline release from pre-loaded rat pancreatic segments. In order to examine the possible intrapancreatic effects of NO, this study applied the NO donor SNP. SNP has been previously used as a source of exogenous NO by a number of authors. SNP seemed to inhibit both basal and EFS-evoked amylase output but had no significant effect on the ACh induced amylase output from rat pancreatic segments. Secretory effects of SNP seemed to be depending on extracellular Ca^{++} concentration. In high Ca^{++} medium, SNP failed to inhibit EFS-evoked amylase output. The above effects of SNP could be mimicked by the permeant cGMP analogue 8-Br cGMP. In fura-2 AM loaded pancreatic acinar cells, both SNP and 8-Br cGMP induced a decrease in basal $[Ca^{++}]_i$ but had no significant effect on the ACh-evoked rise in $[Ca^{++}]_i$.

In contrast to the NO donor SNP, the NOS substrate L-Arg and the NOS inhibitor LNNA had no significant effects on either basal or EFS-evoked amylase output from rat pancreatic segments. It seems probable that in our *in vitro* pancreatic model neither L-Arg nor LNNA (at concentrations investigated) could significantly modify the endogenous (enzymatic) NO production and cause subsequent measurable changes in amylase output.

In summary, the results of this study suggest a dual action of exogenous NO in the isolated rat pancreas. On one hand, NO can diffuse into the acinar cells to inhibit basal amylase secretion. On the other hand, NO may exert neuromodulation (prejunctional inhibition) in the predominantly cholinergic nerve terminals causing a decrease in EFS-evoked ACh release followed by a reduction in the nerve-mediated pancreatic amylase secretion. Our results show that the above neuromodulatory effect of SNP is determined by extracellular Ca^{++} concentration. In isolated acinar cells, both SNP and 8-Br cGMP induced a decrease in basal $[Ca^{++}]_i$. The observations above may suggest that NO can modulate ACh release possibly by inhibiting Ca^{++} influx into cholinergic nerve terminals. Both intrapancreatic effects of NO seem to be mediated by cGMP but the precise intracellular mechanisms remain to be elucidated.

Recent years have seen an explosion of interest in NO in different biological systems leading to the involvement of this molecule in a wide range of functions. However, compared with other tissues, our knowledge ragarding the action of NO in the exocrine pancreas still seems confusing and even contradictory. Further research is clearly required in order to improve our understanding of NO modulation of pancreatic secretory function. Moreover, although the bulk of evidence suggests a crucial role of cGMP as the main intracellular mediator in this tissue, additional candidate mechanisms may exist.

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References

- *Articles*

(I) Adeghate E, **Ember Zs,** Donáth T, Pallot DJ, Singh J (1996) Immunohistochemical identification and effects of atrial natriuretic peptide, pancreastatin, leucin-enkephalin and galanin in the porcine pancreas. *Peptides* 17(3): 503-509.

(II) **Ember Zs,** Fehér E, Donáth T (1997) A nitrogén-oxid szintáz immuncitokémiai kimutatása patkányok hasnyálmirigy szöveteiben. (In Hungarian) *Orv Hetil* 138: 2113-15.

(III) **Ember Zs** (1997) Age-dependent distribution of nitric oxide synthase containing elements in the rat pancreas. *Biogenic Amines* 13(4): 277-284.

(IV) **Ember Zs,** Réti A, Fehér E (2000) Enzyme- and immunohistochemical localization of nitric oxide synthase in nerves of the porcine pancreas. *Neuroscience Letters* 292: 163-166.

(V) Yago MD, Manas M, **Ember Zs**, Singh J (2001) Nitric oxide and the pancreas: Morphological base and role in the control of the exocrine pancreatic secretion. *Molecular and Cellular Biochemistry* 219: 107-120.

(VI) **Ember Zs,** Yago MD, Singh J (2001). Distribution of nitric oxide synthase and secretory role of exogenous nitric oxide in the isolated rat pancreas. *International Journal of Pancreatology* 29: 9-16

- *Citable abstracts*

Ember Zs, Fehér, E, Donáth T (1996) Nitric oxide immunocytochemistry of the pancreas of neonatal and adult rats. *Zeitschrift für Gastroenterologie* 5/1996: 308.

Ember Zs, Yago MD, Singh J (1998) Nitrergic nervous control of amylase secretion and calcium mobilization in the isolated rat pancreas. *J Physiol (London)* 511P: 24P.

Ember Zs, Réti A (2000) Immunohistochemical detection of peripherin in porcine pancreatic neurons. *Zeitschrift für Gastroenterologie* 5/2000: 403.

Yago MD, Manas M, **Ember Zs**, Singh J (2000) Modulation of cholinergic neurotransmission by exogenous nitric oxide in isolated rat pancreatic segments. *J Physiol (London)* 526 P: 21-22P.