Secretagogue-Stimulated Pancreatic Secretion is Differentially Regulated by Constitutive Nitric Oxide Synthase Isoforms in Mice.

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ABSTRACT

Nitric oxide (NO) and NO synthase (NOS) play controversial roles in pancreatic secretion. NOS inhibition reduces CCK-stimulated in vivo pancreatic secretion, but it is unclear which NOS isoform is responsible because NOS inhibitors lack specificity and three NOS isoforms exist: neuronal- (nNOS), endothelial- (eNOS) and inducible- (iNOS). Mice having individual NOS gene deletions were used to clarify the NOS species and cellular interactions influencing pancreatic secretion. In vivo secretion was performed in anesthetized mice by collecting extra-duodenal pancreatic duct juice and measuring protein output. Non-selective NOS blockade was induced with L-NNA (10 mg/kg). In vivo pancreatic secretion was maximal at 160 pmol/kg/h CCK-8 and was reduced by NOS blockade (45%) and eNOS deletion (44%). Secretion was unaffected by iNOS deletion, but increased by nNOS deletion (91%). To determine whether the influence of NOS on secretion involved non-acinar events, in vitro CCK-8 stimulated secretion of amylase from isolated acini was studied and found to be unaltered by NOS blockade and eNOS deletion. The influence of NOS on in vivo secretion was further examined with carbachol. Protein secretion, which was maximal at 100 nmol/kg/h carbachol, was reduced by NOS blockade and eNOS deletion but unaffected by nNOS deletion. NOS blockade by L-NNA had no effect on carbachol-stimulated amylase secretion in vitro. Thus constitutive NOS isoforms can exert opposite effects on in vivo pancreatic secretion. eNOS likely plays a dominant role, since eNOS deletion mimics NOS blockade by inhibiting CCK-8 and carbachol-stimulated secretion, while nNOS deletion augments CCK-8 but not carbachol-stimulated secretion.

Key Words: Nitric oxide, cholecystokinin, carbachol
INTRODUCTION

Nitric oxide (NO), a gaseous neurotransmitter and paracrine regulator, influences diverse processes in the gastrointestinal tract, especially motility, inflammation and neurotransmission. In the pancreas NO may regulate blood flow (24, 41) and exocrine secretion (13, 23, 24, 37, 41, 51) and has related effects on endocrine pancreas insulin secretion (23, 43-45) and peripheral tissue insulin signaling (2, 45, 46). Prior studies have demonstrated that non-selective, pharmacologic inhibition of nitric oxide synthase (NOS) reduces stimulated in vivo pancreatic secretion by cholecystokinin (CCK) analogues in rat (25, 37, 51), pigs (13), cats (41), dogs (24) and humans (23), but the effect of NOS inhibition on basal secretion is less clear and species dependent. The localization of constitutive NOS isoforms in the exocrine pancreas is controversial as is the effect of NOS inhibition on in vitro pancreatic secretion; one study reported that NOS inhibition reduces carbachol-stimulated amylase secretion from pancreatic acini (54) while others showed no effect on CCK-8 or carbachol-stimulated in vitro secretion (20, 24, 25, 37, 57). Regarding in vivo pancreatic secretion, it remains unclear which NOS isoform and what mechanism is responsible for the inhibitory effect of NOS blockade, because three NOS isoforms exist, NOS inhibitors lack high specificity, and NO exhibits complex biological effects. For this reason, use of mice with targeted deletion of individual NOS isoforms (15, 16, 28, 33, 47) is particularly useful to define the biologic role of specific NOS isoforms in pancreatic secretion.

The effects of NO are strongly influenced by the site of NO production, the amount of NO produced, and the specific NOS isoform involved in NO synthesis (35). Each of the three NOS isoforms was named for their initial localization in vascular endothelium (eNOS), neurons (nNOS) and macrophages (iNOS), and they primarily regulate vascular tone, neurotransmission and immune defense, respectively. In the pancreas, the constitutive NOS isoforms (eNOS and nNOS) have been localized to endothelial cells (eNOS), neurons (nNOS) and islets (nNOS), but it is controversial whether either is present in exocrine cells. Many NOS inhibitors are available, but none is
completely selective for one enzyme. Conceivably, one or more NOS isoforms could influence pancreatic secretion; nNOS in either the brain or pancreas could regulate neurotransmission and eNOS could affect pancreatic blood flow.

To clarify the effect of NO on pancreatic secretion we first developed an *in vivo* murine model to establish normal values of pancreatic secretion. Using maximal stimulatory doses of CCK-8 (160 pmol/kg/h) and carbachol (100 nmol/kg/h), we examined the effect of nonspecific NOS inhibition or genetic deletion of individual NOS isoforms on *in vivo* murine pancreatic secretion. Because the localization of NOS isoforms in the pancreas is controversial, we functionally assessed whether acinar events alone were sufficient to explain the effect of NOS on pancreatic secretion by examining secretagogue-stimulated amylase release from isolated pancreatic acini (52).
MATERIALS AND METHODS

Materials. The non-selective NOS inhibitor \( N^\omega \)-Nitro-L-arginine (L-NNA) was obtained from Cayman Chemical (Ann Arbor, MI), collagenase (CLSPA grade) from Worthington Biochemicals (Freehold, NJ), cholecystokinin octapeptide (CCK-8) from Research Plus (Bayonne, NJ). All other reagents were from Sigma.

Animal care and selection. All experiments were approved by the University of Michigan Committee on Use and Care of Animals. All mice were obtained from Jackson Laboratory. Animals were 6- to 8-weeks old, weighed 18-24 g and were maintained in a climate–controlled room kept at 22\(^\circ\) C, exposed to a 12-h light-dark cycle, and fed standard laboratory chow and given water ad libitum. The effect of selective NOS deletion on experimental \textit{in vivo} and \textit{in vitro} pancreatic secretion was studied in mice with targeted gene deletion of nNOS (15), eNOS (47), and iNOS (28). The genetic background for eNOS and iNOS knockout mice was C57BL/6J and for nNOS knockout mice was B6129SF2/J. Control mice were age and sex matched and of identical genetic background.

\textit{In vivo pancreatic secretion.} Mice were anesthetized with a combination of ketamine 80 mg/kg IP plus xylazine (Rompun\textsuperscript{®}) 5 mg/kg IP to produce 30-45 minutes of anesthesia, redosing with 1/3 the original calculated dose of ketamine, but without xylazine. A surgical, 4X-magnifying, Leica MZ6 stereo-microscope was used for microsurgery procedures. A PE-10 cannula was inserted into the right internal jugular vein for secretagogue infusion. A superficial, 1.5-2.0 cm midline, abdominal incision was made, and a PE-10 catheter was inserted into the extra-duodenal, intrapancreatic common bile-pancreatic duct. The abdominal wound was covered with saline gauze and the mouse was placed on a protective pad, overlying a heating pad, to maintain body
temperature at 37 degrees C.

Experiments began following a 30 minute stabilization period. Combined bile-pancreatic (mixed) juice was collected every 15 minutes. In separate experiments, selective bile duct cannulation was performed to assess the relative contribution of bile- to mixed juice protein output. Juice volume was measured and protein concentration was determined spectrophotometrically using protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Basal protein output was obtained from the second of two 15 min baseline, mixed juice samples. The secretagogue intravenous infusion rate was set at 100 µl/h, using a Harvard PHD 2000 Push/Pull Syringe Pump, and dose response studies were performed with CCK-8 (40-400 pmol/kg/h) and carbachol (50-500 nmol/kg/h) using 0.9% sodium chloride as a control vehicle. Protein output (µg/min) was expressed as % basal and statistical analysis was performed on the pooled peak CCK-8 or carbachol response between 45-75 minutes. Secretagogue doses found to produce maximal protein output were used to determine the effect of non-selective NOS blockade and selective NOS deletion on pancreatic secretion. Blood pressure was measured using a Capto SP844 Physiologic Pressure Transducer and PowerLab Data Acquisition Instrument (www.ADInstruments.com).

**In vitro pancreatic amylase secretion.** Pancreatic acini were prepared by collagenase digestion (6, 52) of mouse pancreases and suspended in HEPES-Ringer’s buffer with Eagle’s minimal essential amino acids, 1 mg/ml BSA, 0.1 mg/ml SBTI (soybean trypsin inhibitor), and equilibrated with 100% O2. Viability of acini was >95% based on trypan blue exclusion. Amylase secretion was determined by incubating dispersed pancreatic acini at 37° C for 30 minute with graded concentrations of CCK-8 or carbachol. Following centrifugation, amylase activity in the supernatant and total acinar pellet was determined using Phadebas reagent (Pharmacia Diagnostics, Atlanta, GA) and amylase secretion was expressed as % of total content.
Non-selective NOS inhibition. In vivo, C57BL/6J mice were pretreated with a single IP injection of L-NNA (10 mg/kg) in 0.9% sodium chloride (pH 7.4) 30 minutes prior to beginning CCK-8 or carbachol stimulation. L-NNA (N\textsuperscript{\textalpha}-Nitro-L-arginine) is the stable, active hydrolysis product of L-NAME (N\textsuperscript{\textalpha}-Nitro-L-arginine Methyl Ester) and is known to inhibit all NOS isoforms, and allow for relatively constant NOS inhibition in vivo (1-20 mg/kg IV or IP) and in vitro (0.1-1.0 mM) (12). This L-NNA dose is similar to doses used previously to non-selectively inhibit rat pancreatic NOS (1, 31). The control group received an equal volume of 0.9% sodium chloride. In vitro, dispersed pancreatic acini from C57BL/6J mice were preincubated 30 minutes with L-NNA (10\textsuperscript{-4} M), a dose similar to that used in other cell types (5), prior to incubation with secretagogues.

Statistical analysis. The data reported represent mean and SE from multiple determinations obtained from three or more experiments. Statistical comparisons were performed with the Student’s t-test when comparing only two groups. When comparing three or more groups ANOVA was used followed by post hoc testing with Fisher’s protected least significant differences test (PLSD) using Stat View software (SAS Institute, Cary, NC). Statistical significance was assumed for \( P < 0.05 \).
RESULTS

**CCK-8 and carbachol stimulate in vivo pancreatic secretion dose dependently.** Continuous CCK-8 administration produced a dose dependent increase in *in vivo* bile pancreatic protein output in C57BL/6J mice (Fig. 1A). Basal bile-pancreatic protein output was determined from a single 15 minute juice collection between 15-30 minutes and averaged 4.46 ± 0.34 µg/min pooled from CCK-8 and carbachol stimulation experiments. CCK-8 had no effect at 40 pmol/kg/h and maximally stimulated protein output at 80 and 160 pmol/kg/h to 313 ± 36% of basal; 400 pmol/kg/h CCK-8 induced a lesser response. Continuous carbachol administration produced a dose dependent increase in bile pancreatic protein output in C57BL/6J mice (Fig. 1B). Maximal carbachol-stimulated bile-pancreatic protein output occurred at 100 and 200 nmol/kg/h and was 315 ± 54% of basal. The 160 pmol/kg/h CCK-8 and 100 nmol/kg/h carbachol infusion rates subsequently were used to study the behavior of maximal secretagogue-stimulated bile-pancreatic and bile protein output.

In bile duct cannulation experiments the average basal bile protein output was 1.75 +/- 0.43 µg/min, or 40% of basal mixed juice protein, pooled from CCK-8 and carbachol experiments. Neither carbachol nor CCK stimulation affected bile protein output (Figures 2A & 2B).

**NOS blockade reduces in vivo stimulated pancreatic secretion.** The effect of NOS blockade on *in vivo* pancreatic secretion was studied in C57BL/6J mice. The dose of L-NNA (10 mg/kg IP) employed is similar to doses used to inhibit rat pancreatic NOS (1, 31) and exerts an observable hemodynamic effect; L-NNA increased the systolic blood pressure in an anesthetized mouse from 85 to 105 mm Hg over 30 minutes, similar to the effect of NOS inhibition in rats (2), cats (41) and humans (24). In this study, L-NNA caused a 45% reduction in the maximal CCK-8 stimulated *in vivo* bile pancreatic protein output from 45-75 minutes (Fig. 3A). A more profound effect of L-NNA on secretion was observed during the first 15 minute stimulation period when secretion above basal was nearly abolished. From 30-90 minutes, L-NNA reduced CCK-8 stimulated secretion by 52%. L-NNA similarly inhibited maximal
Carbachol-stimulated \textit{in vivo} bile-pancreatic output, which was reduced by 40\% between 45-75 minutes (Fig. 3B). In addition, the reduction in carbachol-stimulated secretion invoked by L-NNA at both 30-45 and 30-90 minute intervals paralleled that observed with CCK-8 stimulation. Basal bile-pancreatic protein output and basal bile protein output were unaffected by L-NNA; basal bile-pancreatic protein output averaged 5.12 \pm 0.74 \, \mu g/min pooled from CCK-8 and carbachol data (n=13) and basal bile protein output averaged 1.27 \pm 0.18 \, \mu g/min, which was 25\% of basal bile-pancreatic protein output.

\textbf{Constitutive NOS isoforms differentially affect CCK-8 stimulated \textit{in vivo} pancreatic secretion.} To determine the NOS isoform responsible for the inhibitory effect of L-NNA on CCK-8 stimulated \textit{in vivo} pancreatic secretion, similar experiments were performed in mice with individual gene deletions for eNOS, nNOS and iNOS along with age and sex matched controls. Similar to the inhibitory effects of NOS blockade, eNOS deletion caused a 44\% reduction in CCK-8 stimulated \textit{in vivo} bile-pancreatic protein output with a larger effect on the initial 15 minute period (Fig. 4A). By contrast iNOS deletion had no effect (Fig. 4B). Somewhat unexpectedly, nNOS deletion increased protein secretion by 91\% throughout the time course of stimulation (Fig. 4C). Basal bile-pancreatic protein output was unaltered by eNOS- and iNOS gene deletion and averaged 4.66 \pm 0.69 \, \mu g/min (n=11) pooled from the CCK-8 and carbachol-stimulated eNOS deleted group and averaged 4.75 \pm 1.05 \, \mu g/min (n=4) pooled from the CCK-8 stimulated iNOS deleted group. Because the nNOS deficient mice and the respective control mice were on a B6129SF2/J background, CCK-8 dose response experiments were also performed with the B6129SF2/J strain. These mice had a similar dose dependent pattern of secretion as the C57BL/6J mice, achieving maximal secretion at 160 pmol/kg/h, although the maximal CCK-8 stimulated protein output was somewhat less than observed for C57BL/6J mice (data not shown). Basal bile-pancreatic protein output was unaltered by nNOS gene deletion and averaged 3.65 \pm 0.59 \, \mu g/min (n=12) pooled from the CCK-8 and carbachol data,
compared to an average of 4.07 ± 0.76 µg/min (n=16) for the B6129SF/2 control mice data pooled from the CCK-8 and carbachol experiments.

**Carbachol-stimulated in vivo pancreatic secretion is reduced by eNOS deletion.** To determine whether the effects of NOS isoform deletion extended to other secretagogues we carried out carbachol-stimulated *in vivo* pancreatic secretion studies in mice with individual gene deletions for the constitutive NOS isoforms, eNOS or nNOS, along with age and sex matched controls. Similar to the inhibitory effects of NOS blockade, eNOS deletion reduced carbachol-stimulated *in vivo* bile-pancreatic protein output by 45% (Fig. 5A). This inhibition was similar to results with CCK-8. By contrast, genetic deletion of nNOS had no statistically significant effect on carbachol-stimulated *in vivo* bile-pancreatic protein output (Fig. 5B), which differed from the augmentation in secretion due to nNOS deletion in response to CCK-8 stimulation (Fig. 4C). As previously noted, basal bile-pancreatic protein output was unaltered by eNOS and nNOS gene deletion.

**NOS blockade and eNOS deletion do not affect in vitro stimulated pancreatic secretion.** Because the intrapancreatic localization of the constitutive NOS species (eNOS and nNOS) is controversial, we studied *in vitro* stimulated pancreatic secretion by dissociated pancreatic acini to determine whether acinar events alone were sufficient to explain the inhibitory effects of NOS blockade and eNOS deletion on *in vivo* pancreatic secretion. In contrast to *in vivo* experiments, NOS blockade (Fig. 6A) and eNOS deletion (Fig. 6B) had no affect on CCK-8 stimulated amylase release from dispersed pancreatic acini. Similarly, NOS blockade had no affect on carbachol-stimulated amylase release from dispersed pancreatic acini (data not shown).
DISCUSSION

In this study we first established normal values of CCK-8 and carbachol-stimulated in vivo murine pancreatic secretion. Secondly, we demonstrated that non-selective, pharmacologic inhibition of NOS significantly reduced the maximal in vivo pancreatic secretory response of CCK-8 and carbachol, but had no effect on in vitro secretion. Thirdly, by studying mice with genetic deletions of individual NOS isoforms, we showed that the constitutive NOS isoforms (eNOS and nNOS) differentially regulate stimulated pancreatic secretion in vivo. eNOS deletion paralleled the inhibitory effect of L-NNA on in vivo CCK-8 and carbachol-stimulated secretion as well as the lack of effect on in vitro CCK-8 stimulated secretion. In contrast, iNOS deletion had no effect and somewhat surprisingly, nNOS deletion augmented CCK-8 but not carbachol-stimulated in vivo secretion.

In vivo secretion experiments performed with C57BL/6J mice showed that both CCK-8 and carbachol dose dependently increased bile-pancreatic protein output, which was maximal at 160 pmol/kg/h CCK-8 and 100 nmol/kg/h carbachol. Both secretagogues showed a similar ~315% increase in stimulated in vivo pancreatic secretion. Unfortunately, a meaningful comparison to other murine studies of pancreatic secretion is not possible because few have been published and they utilize different methodologies, including the method of juice collection, and the drug type, dose and route of delivery (36, 39, 48). Our results of pancreatic secretion, however, are similar to experiments in rats (30, 32). In rats, which lack a gallbladder, there is a similar in vivo dose dependent increase in bile-pancreatic protein secretion in response to IV CCK-8 (30) and no significant increase in bile protein output (32). Despite methodologic differences, it is interesting that our results of bile output are similar to results of two mouse studies that showed no increase in bile-acid output following either carbachol (500 nmol/kg SC) (36) or CCK-8 (1 nmol/kg SC) (48), however members of the same research group showed that a lower dose of CCK-8 (100 pmol/kg SC) significantly increased bile-acid output (36). Thus, the results we obtained of stimulated in vivo bile-
pancreatic and bile protein outputs are generally consistent with the results of others.

Across species (13, 24, 37, 41, 51), including humans (23), NOS inhibition reduces pancreatic protein secretion arising from vagal electrical stimulation (13) and from the peptides CCK (41), caerulein (37, 51), secretin (41), vasoactive intestinal peptide (VIP) (13), and combinations of CCK, caerulein and secretin (23, 24). In the present study, L-NNA reduced CCK-8 and carbachol-stimulated in vivo pancreatic protein secretion, but not in vivo basal pancreatic protein secretion. The latter finding appears to be species dependent since NOS inhibition reduces basal protein secretion in rats (37, 51) but not in dogs (24) and cats (41). In vitro, L-NNA did not affect CCK-8 or carbachol-stimulated pancreatic acinar amylase release, in agreement with most studies performed in rats (20, 24, 37, 57) and dogs (24). We interpret these data as suggesting that non-acinar cell types mediate the functional effects of NOS blockade observed in vivo.

Previous pancreatic NOS localization studies have failed to fully pinpoint the candidate cell types involved in NO-dependent pancreatic protein secretion. Attempts to localize NOS can be affected by tissue fixation techniques (e.g. paraformaldehyde versus acetone) (22, 53), the NOS detection method and the animal species studied. NOS has frequently been visualized by using NADPH-d (nicotinamide adenine dinucleotide phosphate-diaphorase) activity as a histochemical marker, and by using immunocytochemistry with NOS specific antibodies; however NADPH-d activity may be catalyzed by non-NOS enzymes (53).

Pancreatic endothelial cells from diverse species show eNOS staining associated with NADPH-d activity (22, 53), although non-pancreatic endothelium may express nNOS as well (14, 42). Pancreatic neuronal tissue appears to exclusively express nNOS and is associated with NADPH-d activity (22, 53), but in brain, some neuronal cell populations were reported to express eNOS (8). Multiple studies show NADPH-d activity associated with nNOS or nonselective NOS in intrapancreatic ganglia cell bodies and thin varicose nerve fibers with processes running among
acini, ducts, blood vessels, islets and stroma (7, 9, 10, 22, 53). Islet cell NOS activity occurs in isolated mouse pancreatic islets (43), but data on the constitutive expression of islet cell NOS is conflicting (10, 44, 50, 53). Pancreatic acinar cell NOS expression is less clear. Most studies show absence of both NADPH-d activity and NOS (eNOS or nNOS) staining in pancreatic acini of rats (50, 53), dogs (50), humans (53) and pigs (10). Recently both NADPH-d activity and eNOS staining were observed in rat acinar cells by treating unfixed tissue with acetone prior to histo- and immunocytochemistry and using a rabbit polyclonal antibody directed against a different epitope than the mouse monoclonal antibody used by Worl et al. (22). Two additional studies showed nNOS immunoreactivity in acinar cells of bovine (38) and rat (55) pancreas, but without studying NADPH-d activity. Our preliminary western blotting and immunohistochemical studies, employed monoclonal and polyclonal antibodies similar to those used by Konig et al. (22) and utilized the eNOS and nNOS gene deleted mouse pancreas as negative controls (MJ DiMagno, Y Hao, SA Ernst, JA Williams and C Owyang, unpublished observations). Western blotting showed that nNOS is absent from acinar cells and eNOS is either absent or present as only a small fraction of total pancreatic eNOS. Immunohistochemical studies failed to clarify pancreatic NOS expression, since eNOS and nNOS staining in the control pancreas was not significantly altered by selective eNOS or nNOS gene deletion, even though the expected absence of eNOS and nNOS protein expression in the eNOS and nNOS gene deleted mice was found by western blotting. Even though these studies fail to clarify the localization of NOS in the exocrine pancreas, the in vivo and in vitro pancreatic secretion studies provide evidence that regardless of the presence or absence of NOS in acinar cells, the functional impact of NOS on pancreatic secretion involves non-acinar cell types.

NOS may influence carbachol and CCK-8 stimulated in vivo pancreatic protein secretion through affects on the vasculature, the nervous system, the endocrine system or other non-acinar cell types. Tankel et al. hypothesized that reductions in pancreatic microvascular blood flow (PMBF)
can reduce pancreatic secretion (49). NOS inhibition has been shown to reduce in vivo stimulated pancreatic protein secretion and PMBF (24, 41), although the association between secretory and circulatory events was secretagogue dependent (41). In the present study NOS inhibition and specifically eNOS gene deletion reduced in vivo stimulated pancreatic protein secretion but in contrast to prior studies using NOS inhibitors, the present study was performed in mice and examined the effects of single NOS isoforms. Since eNOS plays an important role in endothelial function, modulating vasodilation and blood perfusion of organs (35), it is conceivable that reduced eNOS enzyme activity (by NOS inhibition or eNOS gene deletion) could impair murine PMBF and interfere with tissue oxygen delivery or extraction for cell metabolism, and thereby reduce in vivo stimulated pancreatic protein secretion. How eNOS is regulated in pancreatic secretion is unknown, but it is conceivable that metabolic products of acinar cells affect eNOS activity, reinforcing the notion that acinar and non-acinar interactions occur and are important during pancreatic secretion.

Nonvascular mechanisms may also explain the effect of eNOS on pancreatic protein secretion, since parallel changes in pancreatic secretion and PMBF do not always occur (18, 40, 41). First, secretin (3) and isosorbide dinitrate (56) stimulated in vivo pancreatic protein secretion at much lower doses than required to augment PMBF. A clearer dissociation of pancreatic protein secretion from PMBF has also been observed in multiple unrelated studies. For example, no change in PMBF was reported despite observing that NOS inhibitors reduce secretin-stimulated in vivo pancreatic secretion in cats (41) and that the NO donor sodium nitroprusside increases in vivo pancreatic protein secretion (17, 41). To explain this dissociation between stimulated pancreatic protein secretion and PMBF, and the relationship of NO to both, Patel et al. proposed that NO may exert its principal effect through a non-vasodilatory mechanism, possibly by modulating vagal stimulation of secretory cells (41) or directly stimulating acinar cells. Although an effect on neurotransmission has not been specifically ascribed to the eNOS isoform, this potential role is conceivable based on evidence that eNOS has been detected in CNS neural cells (8), that eNOS derived NO may play a
role as a retrograde neurotransmitter in the CNS (19) and that NO arising from nonneural, eNOS containing cells may diffuse freely between cells to act directly on intracellular targets (27). Without specifically implicating eNOS (or nNOS), the role of NOS in neurotransmission rather than vasodilation was examined by Holst et al. who showed that NOS inhibition reduces vagal-stimulated pancreatic protein secretion in pigs without affecting vasodilatation, which was attributed to the effects of VIP (13), a hormone with potent effects on PMBF and weak activity as a secretagogue (26). In addition, Klein et al. showed that truncal vagotomy in dogs reduced secretin-stimulated in vivo pancreatic secretion without altering PMBF (21).

Independent of vascular and neural mediated effects, NOS inhibition and specifically eNOS gene deletion may influence exocrine pancreatic secretion through effects on insulin resistance and/or insulin release, both of which may be regulated at peripheral and/or CNS locations. In rats, NOS inhibitors administered by IV route induce acute insulin resistance (2) and intracerebroventricular (ICV) administration induces both peripheral insulin resistance and defects in insulin secretion (45). The effect of individual NOS isoforms on insulin resistance (but not insulin secretion) was examined using hyperinsulinemic-euglycemic clamp studies, which showed that insulin resistance was exhibited by nNOS and to a greater extent by eNOS gene deleted mice (46). Because insulin strongly potentiates CCK (4, 29) and secretin (29) stimulated in vivo pancreatic secretion in rats, the presence of insulin resistance may partially explain why eNOS gene deletion reduces stimulated in vivo murine pancreatic secretion. It is less likely that impaired insulin release would explain the effects of eNOS gene deletion on pancreatic secretion since 1) eNOS gene deleted mice have normal baseline glucose and insulin levels (46); 2) constitutive expression of nNOS rather than eNOS has been identified in islet cells (10, 44, 50); and 3) because islet cells appear to withhold insulin upon exogenous (i.e. eNOS generated) NO stimulation (43), but release insulin upon endogenous (nNOS generated) NO stimulation (43, 44).

In contrast to eNOS, the genetic deletion of the nNOS isoform increased stimulated in vivo
pancreatic secretion. This effect is more likely mediated by altered peripheral or central neurotransmission. nNOS derived NO plays a role in CNS neurotransmission and may play an independent or integrated role in the peripheral nervous system neurotransmission, specifically in the pancreas, where nNOS is expressed in many postganglionic neurons (7, 9, 10, 22, 53), which are nitrergic and cholinergic. The effect of nNOS gene deletion on pancreatic secretion may allow enhanced nerve terminal acetylcholine release during CCK-8 but not carbachol-stimulated in vivo pancreatic protein secretion. Support for this hypothesis arises from 2 studies (11, 34). In a non-pancreatic murine study NOS inhibition and specifically nNOS gene deletion enhanced the electrically evoked release of acetylcholine from “ex-vivo” terminal ileum myenteric plexus-longitudinal muscle preparations (34), leading to enhanced cholinergic muscular contractions. Investigation of such an effect in pancreas segments showed that the NO donor sodium nitroprusside as well as 8-Br cGMP inhibited electrically evoked release of amylase in a calcium-dependent manner and failed to inhibit ACh induced amylase secretion (11).

In summary, these studies provide evidence that non-selective NOS inhibition and eNOS gene deletion reduce CCK-8 and carbachol-stimulated in vivo pancreatic secretion, by modulating non-acinar cell events. In contrast, nNOS gene deletion augments CCK-8 but not carbachol-stimulated in vivo pancreatic secretion, suggesting that eNOS plays a dominant stimulatory role and that nNOS plays a minor role, possibly acting as a negative feedback mechanism. Because of undisputed immunohistochemical localization of eNOS to the vasculature and nNOS to pancreatic nerves, it seems most likely that eNOS acts on pancreatic microvasculature to increase blood flow and that nNOS tonically inhibits acetylcholine release from pancreatic neurons. Further studies are necessary to focus more directly on these loci.
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**FIGURE LEGENDS**

**Fig. 1.** Dose response of *in vivo* CCK-8 (*A*) and carbachol (*B*) stimulated pancreatic secretion. Bile-pancreatic protein output was collected every 15 min, basal output was determined from 15-30 min, and IV secretagogue was infused starting at 30 min and ending at 90 min. Secretagogue-stimulated bile-pancreatic protein output is expressed as a % basal, which averaged 4.18 ± 0.44 µg/min (n=20) pooled from CCK-8 data and 4.69 ± 0.44 µg/min (n=19) pooled from carbachol data. Data points are shown as flat lines, reflecting sample collection over 15 minute intervals. Statistical analysis was performed on the pooled peak secretagogue response between 45-75 minutes and is shown in the upper right bar graph. Data shown are the means and SE of 4 mice per group. *P < 0.005 vs 0 (saline group).

**Fig. 2.** Effects of CCK-8 (*A*) and carbachol (*B*) on *in vivo* bile protein output. Selective bile duct cannulation was performed and fluid was collected every 15 minutes. Basal protein output was determined from 15-30 min, and IV secretagogue was infused starting at 30 min and ending at 90 min. Secretagogue-stimulated bile protein output is expressed as a % basal, which averaged 1.75 +/- 0.43 µg/min, pooled from CCK-8 and carbachol experiments. Data presentation and statistical analysis is similar to that specified for Fig. 1. Data shown are the means and SE of 3 mice per group. *P < 0.05 vs basal group.

**Fig. 3.** Effect of NOS blockade on CCK-8 and carbachol-stimulated *in vivo* bile-pancreatic protein output. Mice were pretreated with L-NNA (10 mg/kg body weight, IP) 30 minutes prior to beginning bile pancreatic juice collection. Juice was collected every 15 min, basal output was determined from 15-30 min, and IV CCK-8 or carbachol was infused starting at 30 min and ending at 90 min. Stimulated bile-pancreatic protein output is expressed as a % basal. Data presentation and statistical analysis is similar to that specified for Fig. 1. Data shown are the means and SE of 4-5 mice per group.
group. *P < 0.005 vs saline group. †P < 0.05 vs control group. ‡P < 0.01 vs control group.

Fig. 4. Effect of selective eNOS- (A) nNOS- (B) and iNOS (C) gene deletion on CCK-8 stimulated in vivo bile-pancreatic protein output. Bile-pancreatic protein output was collected every 15 min, basal output was determined from 15-30 min, and IV CCK-8 was infused starting at 30 min and ending at 90 min. CCK-8 stimulated bile-pancreatic protein output is expressed as a % basal. Basal bile-pancreatic protein output was unaltered by selective NOS gene deletion (see text). Data presentation and statistical analysis is similar to that specified for Fig. 1. Data shown are the means and SE of 4-6 mice per group. *P < 0.01 vs saline group. †P < 0.05 vs control group. ‡P < 0.01 vs control group.

Fig. 5. Effect of selective eNOS- (A) and nNOS- (B) gene deletions on carbachol-stimulated in vivo bile-pancreatic protein output. Bile-pancreatic protein output was collected every 15 min, basal output was determined from 15-30 min, and IV carbachol was infused starting at 30 min and ending at 90 min. Carbachol-stimulated bile-pancreatic protein output is expressed as a % basal. Basal bile-pancreatic protein output was unaltered by selective NOS gene deletion (see text). Data presentation and statistical analysis is similar to that specified for Fig. 1. Data shown are the means and SE of 5-8 mice per group. *P < 0.01 vs saline group. †P < 0.05 vs control group.

Fig. 6. Influence of NOS on CCK-8 stimulated pancreatic acinar amylase release. Dispersed pancreatic acini were prepared from C57BL/6J (A) or eNOS deleted and control mice (B) and were incubated with graded CCK-8 concentrations (A & B). The effect of NOS inhibition was studied by pre-incubating acini (C57BL/6J) in the presence or absence of L-NNA (10^-4 M) (A) and adding fresh
L-NNA during the CCK-8 stimulation period. Stimulated amylase release was measured over a 30 min period. Results are shown as means and SE for 3 independent experiments.
Fig. 1

A

Start CCK (pmol/kg/h)

Protein Output (% Basal)

Time (Minutes)

B

Start Carbachol (nmol/kg/h)

Protein Output (% Basal)

Time (Minutes)
Fig. 2

A

Start CCK (160 pmol/kg/h)

B

Start Carbachol (100 nmol/kg/h)
Fig. 3

A. Start CCK (160 pmol/kg/h)

B. Start Carbachol (100 nmol/kg/h)
Fig. 4

A

Start CCK (160 pmol/kg/h)

Protein Output (% Basal)

Time (Minutes)

B

Start CCK (160 pmol/kg/h)

Protein Output (% Basal)

Time (Minutes)

C

Start CCK (160 pmol/kg/h)

Protein Output (% Basal)

Time (Minutes)
Fig. 5

(A) Start Carbachol (100 nmol/kg/h) at 45-75 minutes.

(B) Start Carbachol (100 nmol/kg/h) at 45-75 minutes.
Fig. 6

A

Amylase Release (% Total)

L-NNA
Control

Basal -12 -11 -10
CCK (Log M)

B

Amylase Release (% Total)

eNOS KO
Control

Basal -12 -11 -10 -9 -8
CCK (Log M)