Endothelial nitric oxide synthase is protective in the initiation of caerulein-induced acute pancreatitis in mice

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DiMagno, Matthew J., John A. Williams, Yibai Hao, Stephen A. Ernst, and Chung Owyang. Endothelial nitric oxide synthase is protective in the initiation of caerulein-induced acute pancreatitis in mice. Am J Physiol Gastrointest Liver Physiol 287: G80–G87, 2004.—The effect of inhibiting nitric oxide (NO) synthase (NOS) or enhancing NO on the course of acute pancreatitis (AP) is controversial, in part because three NOS isoforms exist: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). We investigated whether inhibition or selective gene deletion of NOS isoforms modified the initiation phase of caerulein-induced AP in mice and explored whether this affected pancreatic microvascular blood flow (PMBF). We investigated the effects of nonspecific NOS inhibition with Nω-nitro-L-arginine (L-NNA; 10 mg/kg ip) or targeted deletion of eNOS, nNOS, or iNOS genes on the initiation phase of caerulein-induced AP in vivo and in vitro models. Western blot analysis was performed to assess eNOS phosphorylation status, an indicator of enzyme activity, and microsphere studies were used to measure PMBF. L-NNA and eNOS deletion, but not nNOS or iNOS deletion, increased pancreatic trypsin activity and serum lipase during the initiation phase of in vivo caerulein-induced AP. L-nNOS and eNOS deletion did not affect trypsin activity in caerulein-hyperstimulated isolated acini, suggesting that nonacinar events mediate the effect of NOS blockade in vivo. The initiation phase of AP in wild-type mice was associated with eNOS Thr495 residue dephosphorylation, which accompanies eNOS activation, and a 178% increase in PMBF; these effects were absent in eNOS-deleted mice. Thus eNOS is the main isoform influencing the initiation of caerulein-induced AP. eNOS-derived NO exerts a protective effect through actions on nonacinar cell types, most likely endothelial cells, to produce greater PMBF.

Acute pancreatitis (AP) in humans is poorly understood, potentially lethal, and predominantly associated with alcohol use and biliary stones (38). With either etiology, the incipient events are incompletely understood but are believed to occur in three phases: triggering events, early acinar events, and later acinar and nonacinar events (36). Major potential triggers include pancreatic ischemia, pancreatic duct obstruction, and premature activation of intracellular pancreatic proteases (38), particularly trypsinogen (36). Intra-acinar trypsinogen activation is important because it is a triggering event, occurring in the setting of hereditary pancreatitis (7, 42), and an early acinar event in multiple experimental models of AP, even among models with disparate morphological patterns of pancreatic injury and mortality (36). Although vasoactive and inflammatory mediators play roles in the later acinar and nonacinar events of AP, these mediators may also affect the early events of AP (14).

Nitric oxide (NO) is a potent pancreatic vasodilator (28), but its role is controversial in experimental AP. NO had a protective effect in several animal studies (24, 41), possibly by increasing pancreatic microvascular blood flow (PMBF), but in other studies, it had no effect (40) or a deleterious effect (6, 22), the latter possibly due to oxidative stress (6) or vasodilation and organ hypoperfusion (22). These differences may be explained by the complex and sometimes paradoxical biological effects of NO acting as a regulatory/anti-inflammatory mediator as well as a cytotoxic/proinflammatory agent (13). NO is derived from three NOS isotypes, each named for their tissue localization: endothelial (eNOS), neurons (nNOS), and macrophages (iNOS) (23). For this reason, mice with genetic deletion of individual NOS isoforms (16, 21, 35) are invaluable tools for defining the biological role of each NOS.

To clarify the effect of NO on the initiation phase of experimental AP, we determined whether pharmacological inhibition or genetic deletion of individual NOS isoforms affected early events in murine experimental AP. Although one or more NOS isoforms could influence AP, just as eNOS and nNOS differentially influence in vivo-stimulated pancreatic secretion (8), we hypothesized that eNOS would have a protective effect on AP by maintaining PMBF. For this purpose, we used a caerulein-hyperstimulation model of AP (20) that is well studied and advantageous for examining very early cellular events, particularly for discriminating between acinar and nonacinar events, by using both in vivo and in vitro models. Although most mechanistic studies have been carried out in the in vivo (9, 12, 20) and complementary in vitro (31) rat models, well-characterized time- and dose-dependent features have been described in the in vivo murine model (15, 26). We focused on the initiation phase of caerulein-induced AP, 30 min after a single, supramaximal intraperitoneal injection of the pancreatic secretagogue caerulein (50 μg/kg body wt), a point when multiple markers of pancreatic injury are established both in vivo and in vitro models but before the onset of an inflammatory response.

Materials and Methods

Materials. Sulfated caerulein, a decapeptide analog of the pancreatic secretagogue CCK, was originally obtained from Research Plus (Bayonne, NJ) and, due to product shortages, later obtained from Bachem (Torrance, CA). The nonspecific NOS inhibitor Nω-nitro-L-arginine (L-NNA; 10 mg/kg ip) was obtained from Bachem (Torrance, CA) and, due to product shortages, later obtained from Research Plus (Bayonne, NJ). The nonselective NOS inhibitor Nω-nitro-L-arginine (L-NNA; 10 mg/kg ip) was obtained from Research Plus (Bayonne, NJ) and, due to product shortages, later obtained from Bachem (Torrance, CA).
arginine (L-NNA) was obtained from Cayman Chemical (Ann Arbor, MI), the trypsin substrate Boc-Glu-Ala-Arg-4-methylcoumaryl-7-amide (MCA) from Peptides International (Louisville, KY), chromatographically purified collagenase from Worthington Biochemicals (Freehold, NJ), type I bovine pancreatic trypsin from Sigma (St. Louis, MO), Eagle’s minimal essential amino acids from Gibco (Grand Island, NY), aprotinin and leupeptin from Boehringer Mannheim (Mannheim, Germany), and nitrocellulose membranes from Schleicher & Schuell (Keene, NH). The enhanced chemiluminescence (ECL) detection system, horseradish peroxidase-conjugated second-
ary antibodies, and X-ray film were from American Biotechnology (Piscataway, NJ). Tris-HCl precast gels and broad-
range prestained SDS-PAGE molecular mass standards were from Bio-Rad (Hercules, CA). Protein A agarose, rabbit polyclonal agarose conjugates [iNOS (sc-650) and eNOS (sc-654)], and the rabbit polyclonal antibodies [anti-nNOS (sc-648), anti-phospho-specific p-eNOS Thr495 (sc-16560)] were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal antibodies [anti-nNOS (N31020), anti-iNOS (N39120), and anti-eNOS (N30200)] and mouse macrophage lysate, human endothelial lysate, and mouse cerebellar lysates (for iNOS-, eNOS-, and nNOS-positive controls) were from BD Biosciences Pharmingen (San Diego, CA).

Animal care and selection. All experiments were approved by the Union Committee on Care and Use of Laboratory Animals. All mice were obtained from Jackson Laboratory. Animals were 6–8 wk old, weighed 18–24 g, and were maintained in a climate-controlled room kept at 22°C, exposed to a 12:12-h light-dark cycle, fed standard laboratory chow, and given water ad libitum. C57BL/6J mice were used for all experimental AP dose-response and time-course experi-
ments and for assessing the effect of pharmacological NOS inhibition. The effect of selective NOS deletion on experimental AP was studied in mice with targeted gene deletion of nNOS (16), eNOS (35), and iNOS (21). 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.

In vivo model of experimental AP. In vivo AP was induced with supramaximal hourly intraperitoneal injections of the pancreatic secretagogue caerulein (50 μg/kg body wt) in 0.9% sodium chloride (pH 7.4), a dose shown to produce maximal pancreatic damage in mice (26); control groups received an equal volume of 0.9% sodium chloride (pH 7.4). This time course of in vivo AP was studied in C57BL/6J mice by injecting caerulein at time zero (first injection) and then hourly up to 7 h (seventh injection). Mice were euthanized at 15, 30, or 60 min after a single injection or 1 h after the last in a series of injections (1, 3, 5, or 7 injections). The initiation phase of caerulein-induced AP was studied 30 min after a single caerulein injection to assess the effects of nonselective NOS inhibition or specific gene deletion of individual NOS isoforms, a time when multiple early markers of AP occur in the in vivo (12) and in vitro (31) rat models.

Serum, tissue preparation, and morphology. Mice were euthanized by carbon dioxide asphyxiation, and mixed arterovenous blood was collected from the decapitated body, centrifuged at 4°C, and serum was stored at −70°C. The pancreas was rapidly removed, weighed, and the development of pancreatic edema was expressed as pancreatic weight as a percentage of body weight. A portion of the pancreas (~50 mg) was snap-frozen in liquid nitrogen and stored at −70°C for later measurement of pancreatic trypsin activity. For morphological studies, a pancreas section was rapidly removed, fixed overnight in 4% neutral phosphate-buffered paraformaldehyde (pH 7.4), embedded in paraffin, sectioned in 3-μm slices, stained with hematoxylin and eosin (H&E), and examined by light microscopy. Sections were coded to mask the experimental group, and morphological changes were analyzed by an experienced morphologist.

In vitro model of experimental AP. Pancreatic acini were prepared by collagenase digestion (8) of pancreases from C57BL/6J mice and suspended in HEPES-Ringer buffer with Eagle’s minimal essential amino acids, 1 mg/ml BSA, and equilibrated with 100% O2. Viability of acini was >95% based on trypan blue exclusion. An in vitro caerulein-hyperstimulation model of AP (31) was performed by incubating dispersed pancreatic acini at 37°C for 30 min in HEPES-Ringer buffer with 100 nM caerulein. After the 30-min incubation period, acini were washed twice with HEPES-Ringer buffer and prepared for measuring intra-acinar trypsin activity.

Nonselective NOS inhibition. As previously described (12), L-NNA is the stable active hydrolysis product of N nitro-L-arginine methyl ester inhibiting all NOS isoforms and providing relatively constant NOS inhibition in vivo (1–20 mg/kg iv or ip) and in vitro (0.1–1.0 mM). In the in vivo AP model, C57BL/6J mice were pretreated with a single intraperitoneal injection of L-NNA (10 mg/kg body wt) in 0.9% sodium chloride (pH 7.4) or control vehicle 30 min before treatment with caerulein. As recently studied and reviewed (8), this dose of L-NNA has been shown to inhibit rat pancreatic NOS and to exert a hemodynamic effect in mice and rats. In vitro dispersed pancreatic acini from C57BL/6J mice were preincubated for 30 min with L-NNA (10−4 M), a dose used to treat acini (8) and other cell types (3) to inhibit NOS.

Assays. Commercially available assays were used for measurement of serum amylase (Phadebas Amylase Test, Pharmacia & Upjohn, Uppsala, Sweden) and serum lipase (Sigma) activities. As described by Saluja et al. (31), supernatant trypsin activity of homogenized fresh acini samples and thawed pancreatic tissue was assayed fluorometrically with the substrate Boc-Glu-Ala-Arg-MCA using excitation and emission wavelengths of 380 and 440 nm. Trypsin activity was expressed as nanograms per milligram of protein, determined using a standard curve for purified trypsin (Sigma). Supernatant protein concentrations were determined spectrophotometrically with the Bio-Rad protein assay (Bio-Rad Laboratories) by measuring absorbance at 595 nm.

To pool data from multiple experiments, data were expressed as percent caerulein-treated controls killed 30 min after one caerulein injection.

PMBF studies. Mice were anesthetized with ketamine (80 mg/kg or 60 mg/kg plus xylazine (5 mg/kg ip; Rompun) to produce 30–45 min of anesthesia, redosing with one-third of the original ketamine dose. The mouse was placed on a protective cover overlying a heating pad to maintain body temperature at 37°C. With the use of a surgical, magnifying (×4) Leica MZ6 stereomicroscope, a 30-cm PE-10 cannula was flushed with heparinized (100 U/ml) sterile saline (BioPal, Wellesley Hills, MA), inserted into the right internal carotid artery, advanced 1 cm toward the aortic arch, secured, and used for micropump insertion. A second PE-10 cannula was similarly prepared and inserted into the right iliac artery for blood sample collection.

With the use of previously described techniques (4), PMBF was measured both before and after the induction of AP. Microspheres (15 μm; Biopal) were infused (80,000/100 μl) over 10 s into the aorta via the internal carotid artery cannula, and a 100-μl blood sample was simultaneously removed over 1 min from the external iliac artery using a Harvard PHD 2000 push/pull syringe pump. The weight of blood samples confirmed the measured volume removal. The total blood removed for two PMBF measurements was 200 μl, equal to 12–16% of circulating blood volume. After euthanasia, pancreas and kidneys were removed, washed in saline, blotted, weighed, dried overnight (75°C), and commercially analyzed by neutron activation for blood flow determination (Biopal). Adequate mixing of microspheres in the arterial circulation was considered when blood flow measurements for both kidneys differed by <10%. Blood flow mea-
surements were expressed as percent basal.

SDS-PAGE and Western blot analysis. Similar to previous descriptions (32), protein lysates were prepared from snap-frozen whole pancreas stored at −70°C or freshly prepared dispersed pancreatic acini. Lysis buffer contained (μM) 50 Tris (pH 7.4), 150 NaCl, 5 EDTA, 25 Na2HPO4, 10 sodium tetraborate, 25 β-glycerophosphate, 1 dithiothreitol, 0.2 sodium orthovanadate, and 1 benzamidine, with 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluo-
ride, and 0.2% Triton X-100. Lysates were briefly centrifuged to remove insoluble debris and were assayed for protein concentration (Bio-Rad). From 250 μg lysate protein, immunoprecipitation was performed using the eNOS and iNOS protein agarose conjugates or using protein A agarose with anti-nNOS or anti-phospho-specific eNOS Thr495 antibodies. Immunoprecipitates were mixed with SDS stop solution (final concentrations 62.5 mM Tris HCl, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromphenol blue, and 5% 2-mercaptoethanol) and boiled for 5 min. Proteins were separated by 7.5% SDS-PAGE, transferred to nitrocellulose membranes, incubated for 1 h with primary monoclonal NOS antibodies (1:500 dilution) and then the respective secondary antibody (1:10,000 dilution), and visualized using the ECL reagent. Quantitation of Western blot analyses was performed using Multi-Analyst software (Bio-Rad).

Statistical analysis. The data reported represent means ± SE from multiple determinations obtained from three or more separate experiments. Statistical comparisons were performed with the Student’s t-test for Western blot analysis data, which compared two groups. For the AP experiments, the effect of both treatment (caerulein) and NOS (inhibition or gene deletion) on a given parameter was analyzed by two-way ANOVA followed by post hoc testing with Fisher’s protected least squares difference using StatView software (SAS Institute, Cary, NC). Statistical significance was assumed for P < 0.05.

RESULTS

Time course of the initiation of caerulein-induced AP. Caerulein hyperstimulation of C57BL/6J mice produced clear evidence of pancreatic injury 30 min after one injection, based on evidence of an early maximal increase in intrapancreatic trypsin activity (Fig. 1A), hyperlipasemia (Fig. 1B), and increases in pancreatic weight (Fig. 1C), a marker of edema. Light microscopic morphological changes were not evident, although actin cytoskeletal disruption was prominent after staining with rhodamine phalloidin (data not shown).

At later time points, other features became evident. Hyperlipasemia (Fig. 1B) increased linearly for 7 h and pancreatic weight (Fig. 1C) increased in a near-linear fashion for 5 h and then remained relatively constant. Hyperamylasemia was significant at 1 h and thereafter increased parallel to lipase (data not shown). Intrapancreatic trypsin activity (Fig. 1A) had an early maximum at 30 min and rapidly declined to near-basal levels. Pancreatic histological changes were observed in a time-dependent manner; the interlobular septa were diffusely expanded and significant perilobular acinar cell damage was present at 5 h (following 5 injections), leukocyte infiltration was first observed at 7 h, and acinar cell vacuolization was not observed in this time frame (data not shown).

NOS blockade worsens caerulein-induced AP during the initial phase. The effect of NOS blockade on the initiation of in vivo caerulein-induced AP was studied in C57BL/6J mice. L-NNA augmented the effect of caerulein on the initiation of in vivo AP by triggering a greater increase in intrapancreatic trypsin activity (Fig. 2A) and serum lipase (Fig. 2B) compared with controls. Caerulein increased pancreatic weight (Fig. 2C), which was unaffected by L-NNA. At this time (30 min), no light microscopic morphologic changes of H&E-stained sections were observed. However,

Fig. 1. Time course of in vivo caerulein-induced acute pancreatitis. Parameters relevant to pancreatitis are shown over a 7-h period, during which mice received hourly intraperitoneal injections of caerulein (50 μg/kg body wt) starting at 0 h. Intrapancreatic trypsin activity is expressed as nanograms per milligram protein and showed an early maximal increase and a rapid decline to near baseline (A). Serum lipase increased in a near-linear fashion (B) and pancreatic weight, expressed as %body wt, increased in a near-linear fashion for 5 h and then remained relatively constant (C). Graphs show the means ± SE of 8–12 animals at each time point. *P < 0.05 vs. control (saline).
caerulein caused redistribution of F-actin, but this was unaffected by l-NNA (data not shown).

eNOS deletion worsens caerulein-induced AP during the initial phase. Only eNOS deletion, but not nNOS or iNOS deletion, affected markers of AP during the initiation of AP. eNOS deletion contributed to a greater increase in intrapancreatic trypsin activity (Fig. 3A) and serum lipase (Fig. 3B) compared with wild-type (WT) mice treated with caerulein. Although pancreatic weight (Fig. 3C) and redistribution of F-actin (data not shown) increased in all caerulein-treated groups, these measures were similar in eNOS knockout and WT groups. Neither nNOS nor iNOS deletion significantly affected intrapancreatic trypsin activity (Fig. 3, D and E) or other markers (data not shown) of AP.

Pancreatic NOS protein expression. Protein expression of the constitutive pancreatic NOS isoforms (eNOS and nNOS) was performed by Western blot analysis using whole pancreas lysates. Pancreatic eNOS and nNOS proteins were present in whole pancreas lysates of control mice but absent in those from eNOS- and nNOS-deleted mice, respectively (data not shown).

Fig. 2. Effects of nitric oxide synthase (NOS) blockade on the initiation phase of in vivo caerulein-induced acute pancreatitis. Mice were pretreated with N\textsuperscript{\textsubscript{\textalpha}}-nitro-L-arginine (l-NNA; 10 mg/kg body wt) and then 30 min later administered a single intraperitoneal injection of caerulein (50 \textmu g/kg) and euthanized after an additional 30 min. Both intrapancreatic trypsin activity (A) and serum lipase activity (B) are expressed as % control group killed 30 min after a single caerulein injection. Measured values for serum lipase following caerulein treatment averaged 199 ± 18 U/l. Pancreatic weight (C) is expressed as % body wt. Each bar represents the means ± SE of saline (n = 4–6) and caerulein (n = 11–12)-treated animals pooled from 3 experiments. *P < 0.001 vs. control (saline). ^P < 0.01 vs. caerulein-treated control (saline).

Fig. 3. Endothelial NOS (eNOS) is the only NOS isoform influencing the initiation phase of caerulein-induced acute pancreatitis. eNOS-, neuronal NOS (nNOS)-, and inducible (iNOS)-deficient mice and control mice were administered caerulein 50 \textmu g/kg ip and euthanized 30 min later. Intrapancreatic trypsin activity (A, D, and E) and serum lipase activity (B) are expressed as a percentage of the control group killed 30 min after a single caerulein injection (described in Fig. 2). C: pancreatic wt, KO, knockout. Each bar represents the means ± SE of saline (n = 4–6) and caerulein (n = 8–12)-treated animals pooled from 3 experiments. *P < 0.001 vs. control (saline). ^P < 0.01 vs. caerulein-treated wild type (WT).
not shown), confirming that the commercially obtained mice were true “knockouts.” Baseline pancreatic eNOS and nNOS expression in C57BL/6j mice also remained unaltered throughout a 7-h caerulein-induced AP time-course experiment (data not shown). iNOS protein, which is not constitutively expressed in the pancreas, was not detected in either control or iNOS gene-deleted mice, either at baseline or during the AP time-course experiment (data not shown).

nNOS protein expression in acinar cell lysates was 9.0 ± 3.1% of total pancreatic nNOS when equal amounts of protein were evaluated (Fig. 4A), this acinar content can most likely be attributed to contamination. In contrast, acinar eNOS protein expression was 30.1 ± 3.1% of total pancreatic eNOS (Fig. 4B), suggesting some eNOS may be present in acinar cells. Immunohistochemical analysis failed to further resolve this issue (data not shown).

In vivo effect of NOS blockade and eNOS deletion on caerulein-induced increases in intrapancreatic trypsin activity involves nonacinar events. Although our previous study showed that eNOS had no functional impact on CCK-stimulated amylase release from dispersed pancreatic acini (8), our Western blot analysis data and a recent immunohistochemistry study (19) raised the possibility that eNOS localizes to pancreatic acinar cells. For this reason, we studied an in vitro model of AP using dissociated pancreatic acini to functionally determine whether acinar events alone were sufficient to explain the in vivo effects of l-NNa and eNOS deletion on intrapancreatic trypsin activity. Similar to published data (31), 100 nM caerulein increased intracellular trypsin activity in dissociated acini. In contrast to in vivo experiments, however, NOS blockade had no effect on acinar trypsin activity (Fig. 5A), and in several experiments, a higher dose of l-NNa (10<sup>-3</sup> M) had no effect (data not shown). Similarly, eNOS deletion had no effect on caerulein-induced increases in acinar trypsin activity (Fig. 5B).

**Fig. 5.** Lack of effect of NOS blockade and eNOS deletion on in vitro caerulein-hyperstimulated pancreatic acinar trypsin activity. Acini were prepared from C57BL/6j (A) or eNOS-deleted and control mice (B) and were incubated 30 min with 100 nM caerulein (A and B). The effect of NOS inhibition was studied by preincubating acini (C57BL/6j) in the presence or absence of l-NNa (10<sup>-3</sup> M) and adding fresh l-NNa during the caerulein incubation period. Caerulein hyperstimulation induced a significant increase in pancreatic acinar trypsin content, expressed as nanograms of trypsin per milligram protein. Baseline and caerulein-induced acinar trypsin activity were unaffected by NOS blockade (A) or eNOS deletion (B). Each bar represents the means ± SE pooled from 3 experiments. *P < 0.05 vs. control (saline).

**Fig. 4.** Pancreatic eNOS protein expression is primarily extra-acinar. Whole pancreas and acinar cell lysates were prepared from the same C57BL/6j mice, and Western blot analysis was performed for eNOS (A) and nNOS (B) proteins (as described in MATERIALS AND METHODS) from an identical amount of total protein. Data shown are the means ± SE of 4 separate experiments and are expressed as % whole pancreas protein expression. *P < 0.001 vs. whole pancreas.

**Fig. 6.** eNOS Thr<sup>495</sup> dephosphorylation occurs during the initiation of AP. Changes in eNOS enzyme activity are heavily influenced by changes in eNOS phosphorylation status (10). Of the two major amino acid phosphorylation sites, Ser<sup>1177</sup> and Thr<sup>496</sup>, the greatest change in eNOS enzyme activity is associated with dephosphorylation of Thr<sup>495</sup>, although regulation of both sites often occurs as a coupled event (10). Studies performed in C57BL/6j mice (WT) showed a baseline PMBF of 0.44 ± 0.08 ml·min<sup>-1</sup>·g<sup>-1</sup>
multiple markers of cell injury during the initial (30 min) phase. Because of these relationships and findings in this study of multiple markers of cell injury during the initial (30 min) phase of AP, the rise in intrapancreatic trypsin activity at this point was most likely attributable to trypsinogen activation.

Experiments using a nonselective NOS inhibitor (L-NNA) provide evidence that NO exerts a protective effect on the initiation phase of AP, when intrapancreatic trypsin activity reaches an early peak. L-NNA enhanced intrapancreatic trypsin activity and serum lipase during in vivo caerulein-induced AP but had no effect on trypsin activity within caerulein-hyperstimulated isolated acini. Similar to the effect of L-NNA, eNOS (but not nNOS or iNOS) gene deletion enhanced intrapancreatic trypsin activity and serum lipase during in vivo caerulein-induced AP but had no effect on trypsin activity within caerulein-hyperstimulated isolated acini. Because L-NNA and eNOS gene deletion had no effect on the actin cytoskeleton during the initiation of AP in vivo, the mechanisms involved in intra-acinar disaggregation of actin may occur independently of those responsible for modulation of intrapancreatic trypsin activity. Combined, we interpret these data as suggesting that eNOS is the sole NOS isoform affecting the initiation phase of caerulein-induced AP and that nonacinar cell types are involved to explain the effects of NOS blockade and eNOS deletion observed in vivo.

The role of NO in experimental AP is controversial. Some studies showed that NO was protective (24, 41), possibly by enhancing PMBF. In other studies, NO had no effect (40) or was injurious (6, 22), possibly by potentiating oxidative stress. In contrast to our study, these studies mostly involved rats and examined the late phases of AP. These studies also did not account for the effects of single NOS isoforms. Two recent studies (5, 29) evaluated the effects of iNOS deletion in mice during the inflammatory stage of AP, but the findings were conflicting.

To understand the impact of individual NOS isoforms on AP, others have performed morphological and functional pancreatic localization studies (19, 43), the latter influenced by NOS detection methods, tissue fixation techniques, and animal species. In diverse species, endothelium had eNOS immuno-

tissue, similar to values observed by others in mice (4) and dogs (2). This increased to 1.22 ± 0.125 ml min⁻¹ g tissue⁻¹ 30 min after caerulein administration, a 178% increase similar to values observed by others in rats (34). Genetic deletion of eNOS had no effect on basal PMBF but nearly abolished the augmentation of PMBF observed in WT mice during AP (Fig. 7).

DISCUSSION

In this study, we first demonstrated in mice that multiple markers of pancreatic injury were evident during the initiation phase of AP, 30 min after supramaximal caerulein stimulation. Second, we demonstrated that the effects of caerulein at this time point were enhanced by nonselective, pharmacological inhibition of NOS, whereas inhibition of NOS had no effect on isolated pancreatic acini in the in vitro model of caerulein-induced AP. Similarly, by studying mice with individual NOS gene deletions, we showed that only eNOS affected the initiation phase of AP but had no effect on the in vitro model of caerulein-induced AP. Finally, the initiation of AP was associated with eNOS Thr⁴⁹⁵ dephosphorylation [an indicator of eNOS activation (10)] and a 178% increase in PMBF, which was almost completely inhibited in eNOS-deleted mice.

Prior murine AP studies showed distinct early initiation and late inflammatory phases, in part defined by biphasic increases in intrapancreatic trypsin activity (15), contrasting with the monophasic pattern in rats (12, 14). Three distinct processes modulate intrapancreatic trypsin activity: trypsinogen activation, trypsin inhibition by trypsin inhibitor, and trypsin degradation. Early increases in trypsin activity and trypsinogen activation occur in parallel (12, 15), and trypsinogen activation and acinar cell injury are closely linked events (12, 14, 15, 31). Because of these relationships and findings in this study of multiple markers of cell injury during the initial (30 min) phase of AP, the rise in intrapancreatic trypsin activity at this point was most likely attributable to trypsinogen activation.

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reactivity and nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) activity (19, 43) and neuronal tissues had nNOS staining and NADPH-d activity (19, 43). Murine islet cell NOS activity was reported (30), but data on constitutive expression of islet cell NOS were conflicting (33, 39). In acini, most studies showed no NOS staining or NADPH-d activity (39, 43), although nNOS staining was reported (without NADPH-d analysis) in bovine (25) and rat (44) acini, and eNOS staining and NADPH-d activity were recently demonstrated in rat acini but varied depending on tissue-fixation technique (19).

To clarify constitutive NOS expression in pancreatic acini, we performed Western blot studies of mouse whole pancreas and acinar preparations, using eNOS and nNOS gene-deleted mouse pancreata as negative controls. Data showed that nNOS was absent from acini and that eNOS was primarily extracrine. This latter finding also was reported by Xu et al. (44) but ascribed to contamination based on immunohistochemical data showing eNOS-expressing capillaries adhering to non-eNOS-expressing acini. Irrespective of eNOS localization in acini, we showed by in vivo and in vitro studies that the functional impact of eNOS on the initiation of AP [and stimulated pancreatic secretion (8)] involved nonacinar events.

In recognition that eNOS modulates vasodilation and blood perfusion of organs (23), studies were performed to assess eNOS regulatory changes and alterations in PMBF during the initiation phase of AP. Changes in eNOS enzyme activity are most heavily influenced by changes in eNOS phosphorylation status and posttranscriptional processes that influence mRNA stability. Because the present study detected no change in pancreatic eNOS protein expression during 7-h AP time-course experiments (data not shown), determination of the eNOS phosphorylation status was considered more relevant to eNOS regulation than examining factors known to influence eNOS mRNA half-life (i.e., LPS, cytokines, and hypoxia) (10). The two major amino acid phosphorylation sites are located within the eNOS calmodulin (CaM) binding sequence (10); dephosphorylation of (constitutively phosphorylated) Thr495 is associated with a 10- to 20-fold increase in enzyme activity and is usually coupled to phosphorylation of Ser1177, which alone is associated with a smaller two- to fourfold increase in activity (10). In the present study, we observed pronounced dephosphorylation of the eNOS Thr495 residue without clear changes in the Ser1177 site (data not shown), although the latter finding was likely influenced by the poor sensitivity of available eNOS phospho-Ser1177 antibodies. To understand regulation of pancreatic eNOS phosphorylation, it will be important in future studies to examine eNOS signal complex assembly and membrane association; both events are required for eNOS phosphorylation and activation and are influenced by eNOS protein-protein interactions (i.e., caveolins and heat-shock proteins) and eNOS posttranslational modifications (i.e., myristolation and palmitoylation) (10).

Acinar events are commonly believed responsible for the initiation of AP (36, 38, 42), but other studies (14) as well as the present study indicate that nonacinar events are also important. A recent study (14) showed that genetic deletion of the neutrophil and endothelial enzyme NADPH oxidase abolished intrapancreatic trypsinogen activation and attenuated pancreatic injury during AP, possibly by suppressing NADPH oxidase-dependent generation of reactive oxygen species (ROS), which have been shown to enhance trypsinogen activation and tissue injury during the early phase of AP (27, 37). In the present study, ROS may also mediate the effect of eNOS deletion or inhibition on intrapancreatic trypsin activity and AP severity, because NO may suppress endothelial and neutrophil NADPH oxidase activity (11). Alternatively, secretory dysfunction, a hallmark of AP (36, 38, 42) and a recently described phenotype of eNOS-deficient mice (8), could explain the effects of eNOS gene deletion on AP, possibly by intracrine concentration of proteinases during AP. Finally, the effect of eNOS deletion on AP may be related to pancreatic hypoperfusion. eNOS has been described as a peripheral chemoreceptor for hypoxia (18), which, in response to AP, may trigger PMBF augmentation to maintain oxygen delivery or oxygen extraction for cell metabolism and to clear injurious by-products of metabolic processes. Functionally, the present study shows that dephosphorylation of eNOS Thr495 was coupled to a 178% increase in PMBF in C57Bl/6J mice, a value similar to rat studies (34) and an effect that was abolished by eNOS gene deletion. Although there is no direct evidence to explain how changes in PMBF affect intrapancreatic trypsin activity, alterations in PMBF were clearly shown by us and others to occur during the early phase of AP (17, 34) and to correlate inversely with the severity of clinical (1) and experimental (17, 34) AP; PMBF decreased in the severe, necrotizing form of pancreatitis but increased in the mild, edematous form (17, 34). For future studies, it remains to be determined whether eNOS activity is responsible for PMBF changes in both mild and severe AP and how the late inflammatory phase of AP is affected by NOS isoforms.

In summary, this study provided evidence that nonselective NOS inhibition and, specifically, eNOS gene deletion increased factors associated with pancreatic injury during the initiation of caerulein-induced AP in mice, principally by modulating intrapancreatic trypsin activity through effects on nonacinar cell types. Because enhanced PMBF was associated with eNOS Thr495 dephosphorylation (an indicator of eNOS activation) during the initiation of AP and augmentation in PMBF was blocked by eNOS gene deletion, it is likely that eNOS is responsible for the alterations in PMBF during the initiation of AP.

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