CHOLECYSTOKININ SECRETAGOGUE-INDUCED GASTROPROTECTION: ROLE OF NITRIC OXIDE AND BLOOD FLOW

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ABSTRACT

BACKGROUND: This study was done to examine the role of CCK in gastric mucosal defense and to assess the gastroprotective roles of nitric oxide and blood flow. METHOD/RESULTS: In rats, the CCK secretagogues, oleate and soybean trypsin inhibitor augmented gastric mucosal blood flow and prevented gastric injury from luminal irritants. Type A CCK receptor blockade negated CCK secretagogue-induced gastroprotection and exacerbated gastric injury from bile and ethanol, but did not block adaptive cytoprotection. CCK secretagogue-induced gastroprotection and hyperemia were negated by non-selective nitric oxide synthase (NOS) inhibition (N⁶-nitro-L-arginine methyl ester) but not by selective inducible NOS inhibition (aminoguanidine). Gastric mucosal calcium-dependent NOS activity, but not calcium-independent NOS activity, was increased following CCK and CCK secretagogues. CONCLUSIONS: The release of endogenous CCK plays a role in the intrinsic gastric mucosal defense system against injury from luminal irritants. The protective mechanism appears to involve increased production of nitric oxide from primarily the constitutive isoforms of NOS and a resultant increase in blood flow.

KEYWORDS: gastric injury, nitric oxide synthase inhibitors, stomach, rats
INTRODUCTION

We and others have previously shown that exogenous cholecystokinin (CCK) prevents macroscopic and morphologic injury to the gastric mucosa caused by a variety of luminal irritants (7,8,16,24,26,27,33). The fact that gastroprotection was observed in doses approaching physiologic significance led us and others to speculate that release of endogenous CCK following ingestion of a meal enabled the gastric mucosa to withstand the damaging effects of luminal irritants, such as bile or ethanol, that often accompany a meal. Konturek et al. later demonstrated that transduodenal oleate had gastroprotective actions against ethanol, and gastroprotection was negated by administration of a Type A CCK receptor antagonist (16). The latter findings suggested that oleate caused the release of endogenous CCK that then exerted its protective actions through activation of Type A CCK receptors, but CCK levels following administration of oleate were not measured. Furthermore, CCK secretagogues differ in how they elicit the release of CCK. For example, food substances such as oleate directly interact with CCK-containing cells in the duodenum to stimulate its secretion (18). In contrast, soybean trypsin inhibitor (SBTI) stimulates release of CCK by inhibiting digestion of two naturally occurring trypsin-sensitive peptides, monitor peptide and “CCK releasing factor” (18). The effects of SBTI on gastric injury and the role of CCK have not been examined. Moreover, the ability of CCK secretagogues to prevent damage from irritants other than ethanol and the mechanism(s) responsible for this protective action remain to be fully elucidated.

In addition to having gastroprotective effects, CCK is a potent vasodilator and has been shown to enhance gastric mucosal blood flow (GMBF) following its administration (11,16,26). The gastric hyperemic response appears to be mediated by nitric oxide because non-selective nitric oxide synthase (NOS) inhibitors have been shown to negate the ability of exogenous CCK to
augment GMBF (11,16). To our knowledge, the effects of NOS inhibition on CCK secretagogue-induced gastroprotection and gastric hyperemia have not been reported. Interestingly, Brzozowski et al. reported that CCK-induced gastroprotection is due to increased nitric oxide production from overexpression of inducible NOS (iNOS or NOS-2) (13). However, gastric hyperemia in response to nitric oxide is derived primarily from endothelial NOS (eNOS or NOS-3), which is associated with mucosal blood vessels (33). Moreover, we recently reported that overexpression of NOS-2 is associated with increased gastric injury and is not gastroprotective (12). For these reasons and the aforementioned considerations, this study was undertaken to test the hypothesis that CCK secretagogue-induced protection is mediated by increased nitric oxide production from the constitutive isoforms of NOS and enhanced GMBF.

To address this hypothesis, we performed four experiments. The first experiment assessed the gastroprotective effects of two different CCK secretagogues against three luminal irritants. The second experiment determined whether these CCK secretagogues increase CCK levels. The third experiment examined the role of Type A and Type B CCK receptors in gastric injury. The fourth experiment evaluated the role of nitric oxide in this process and examined the role of GMBF as a potential protective mechanism. Portions of this work have been published in abstract form (6,23).

**METHODS**

**Animals**

Female Sprague-Dawley rats weighing approximately 200 g were used in all studies and were housed at constant room temperature with 12:12-hour light-dark cycles. Rats were maintained on certified custom formula rat chow ordered in bulk from Harlan Teklad to diminish the
inherent variability in composition due to least cost formulation that could alter basal gut peptide levels. Harlan Teklad Certified Teklad Rodent Diet (W) product number 8728 (Madison, WI) contains 24% crude protein, 4% crude fat, 4.5% crude fiber and provides 3.93 kcals/g gross energy. All experiments were performed in conscious rats deprived of food for 18-24 hours but allowed free access to water. On the day of experimentation, all animals were randomly assigned to one of several groups. All experimental protocols were previously approved by the University of Texas at Houston Animal Welfare Committee before any studies were conducted.

Assessment of CCK Secretagogues against Gastric Injury from Luminal Irritants

Macroscopic Analysis

The first set of experiments was designed to ascertain whether oleate or SBTI, two substances that elicit the release of endogenous CCK by different mechanisms (17,18), could prevent or attenuate gastric injury caused by acidified ethanol, concentrated acid, or concentrated base. The initial studies were undertaken with transduodenal administration of oleate and SBTI because the CCK containing cells are primarily located in the proximal duodenum (4). However, because orogastric administration of these two CCK secretagogues (20) had similar gastroprotective actions as transduodenal administration (see below and Results), the majority of the studies were done with orogastric administration of oleate and SBTI to obviate the necessity of laparotomy and general anesthesia to place transduodenal catheters. Nevertheless, the first set of experiments was performed using duodenal catheters to infuse various nutrients.

In the initial experiment, rats were anesthetized with an intraperitoneal injection of 6 mg/kg xylazine and 70 mg/kg ketamine. After obtaining adequate anesthesia, a midline abdominal incision was made and a duodenal catheter (silicone elastimer, ID 0.030 in, Baxter Scientific
Products) was inserted 5 mm distal to the pylorus and secured with a purse-string suture. This catheter was then tunneled subcutaneously from the abdomen to an exit point along the nape of the neck. Catheters were connected to a dual channel swivel mechanism that permitted free range of motion within the cage (including normal access to food and water) and granted investigators access to the catheters without disturbing the animals. Rats were treated with chloramphenicol (50 mg/kg ip) at the time of surgery and for 3 days post-operatively. Animals were allowed to recover for 7 days after the operation. After recovery, rats were fasted for 18–24 hours and then given a 1 ml transduodenal bolus of oleate (100 mM), SBTI (100 mg), or saline for 30 minutes. This concentration of oleate and dose of SBTI have been shown to increase CCK levels in the rat (17,20). After the 30-minute treatment time, rats were given a 1 ml orogastric bolus of acidified ethanol (150 mM HCl-50% ethanol). Because this concentration of alcohol in combination with hydrochloric acid results in macroscopic damage within 5 minutes, rats were killed 5 minutes after receiving this damaging agent (24,26). The total area of macroscopic injury to the acid-secreting portion of the stomach (where damage routinely occurs) was quantified using computerized planimetry. Damage was reported in mm$^2$ ± SE for each experimental group. A sample size of 7 rats per group was used.

The next set of experiments evaluated the effect of orogastric administration of CCK secretagogues. For this experiment, conscious rats were given a 1 ml orogastric bolus of oleate, SBTI, or saline in the above doses. Following the 30-minute treatment time, rats were given acidified ethanol for 5 minutes and macroscopic injury was determined. In order to determine the onset of gastroprotection following administration of a CCK secretagogue, rats were given SBTI orogastrically, and the stomach was challenged with acidified ethanol at 5, 10, or 15 minutes. In additional rats, using similar treatment groups, the exposure to acidified ethanol was
extended to 1 hour to examine whether CCK secretagogues prevented gastric injury from longer periods of exposure to the luminal irritant or simply delayed the onset of injury. Because this set of experiments demonstrated that orogastric administration of CCK secretagogues was as efficacious as transduodenal administration, the remaining experiments were conducted with orogastric administration of oleate, SBTI, and saline.

In order to evaluate the effects of CCK secretagogues against other luminal irritants, rats were treated with orogastric saline, oleate, or SBTI for 30 minutes and then given a 1 ml orogastric bolus of either concentrated acid (0.75 N HCl) or concentrated base (2N NaOH). Rats were killed 5 minutes after receiving the damaging agent and macroscopic injury determined.

**Morphologic Analysis**

In a separate set of experiments using a similar protocol, the morphological correlates of the resultant injury or protection were determined in animals receiving a 30 minute treatment with either orogastric saline or oleate. In these studies, rats were killed 5 minutes after exposure of the stomach to acidified ethanol (n = 5/group). Stomachs were removed and fixed in Karnovsky’s fixative (14) as previously reported (25). After fixation, each stomach was opened along the lesser curvature and sectioned by an independent observer blinded to the protocol (MS; see acknowledgements). Sections (2 x 10 mm) of glandular mucosa were stained with hematoxylin and eosin and processed for routine microscopy utilizing standard techniques. Gastric mucosal damage was assessed by previously published criteria, as follows: Type I damage, involvement of luminal surface mucus cells only; Type II damage, involvement of luminal surface and gastric pit mucus cells; Type III damage, involvement of surface and gastric mucus cells as well as upper gland cells; Type IV damage, severe injury to all surface and all or
most of glandular epithelium (24,26). After the degree of damage had been assessed the code was broken for the treatment group.

*Assessment of CCK Secretagogues on GMBF*

In this set of experiments, the effects of transduodenal saline, SBTI, oleate, or intravenous CCK on GMBF over time were assessed with laser Doppler (25). Rats were anesthetized with an intraperitoneal injection of xylazine and ketamine. After induction of anesthesia, the stomach was exposed by a mid-line incision. Through the non-glandular forestomach, a catheter was introduced to provide access for a Teflon-coated laser optic flow probe (PeriFlux PF 409, Standard Probe, 0.25-mm fiber separation). The flow probe was positioned to allow contact with the glandular or acid-secreting portion of the stomach. After appropriate position of the probe was insured, the stomach was allowed to equilibrate for 30-minutes. Following equilibration, mucosal blood flow to the stomach was recorded continuously with a laser-Doppler flow monitor (PeriFlux 4001 Masters; Perimed, Jäärnfälla, Sweden). Blood flow was recorded for a 5-minute period as the measurement of basal GMBF. Following basal GMBF determinations, a 1 ml injection of transduodenal saline, oleate (100 mM), or SBTI (100 mg) was given and blood flow recorded continuously for 60 minutes. In additional rats, exogenous CCK (5 nmol/kg iv) was given to compare the effects of CCK secretagogues against exogenous CCK-induced changes in GMBF. We also examined GMBF after the luminal irritant acidified ethanol in the presence and absence of the CCK secretagogue SBTI. In all treatment groups, the peak blood flow was averaged in each rat and the average of each group was reported as percentage of values recorded in control rats. Each treatment group consisted of 5 or more animals.
Effects of Orogastric Oleate and SBTI on Plasma CCK Levels

For this experiment, conscious rats were given a 1 ml orogastric bolus of saline, oleate (100 mM), or SBTI (100 mg). After 30 minutes, rats were killed and blood was obtained in heparinized syringes and immediately placed on ice in glass tubes containing 15 units/ml sodium heparin (Liquaemin), and 100 units/ml aprotinin (Trasylol). The plasma was separated by centrifugation (1000g, 10 minutes, 4°C) and stored at -20°C for CCK radioimmunoassay (RIA). The RIA for CCK was developed in the laboratory of Dr. George Greeley, who provided the technical expertise in performance of this assay. This sensitive and specific RIA utilized CCK-8 N-terminus specific antiserum OAL 656 as previously reported (13). Using 125I-Bolton-Hunter labeled CCK-8, the relative potencies of CCK-8 and CCK-33 were 100% and 67.8% respectively. Cross-reactivity to sulfated gastrin-17 was 0.18% and the anti-serum did not bind to CCK-4, non-sulfated CCK-8, or non-sulfated gastrin-17. In these experiments, CCK was extracted from plasma by adsorption onto C18 SEP-PAC cartridges previously washed with 5 ml of methanol and 20 ml of water according to techniques described by Liddle et al. (19).

In addition to assessing the effect of the two CCK secretagogues on plasma CCK levels, a gastroprotective dose of exogenous CCK-8 was also given to compare the magnitude of CCK levels induced by all three agents. Thus, rats were given intravenous saline or CCK-8 (5 nmol/kg) for 10 minutes using a sample size of 5 rats/group. Ten minutes after receiving intravenous saline or CCK, rats were killed and plasma obtained for CCK determinations.

Effects of CCK Receptor Antagonists on CCK Secretagogue Induced Gastroprotection

The role of Type A and Type B CCK receptors in CCK secretagogue-induced gastroprotection was assessed with the selective Type A CCK receptor antagonist, L-364,718 and the selective
Type B CCK receptor antagonist, L-365,260. The treatment times and dosages of the Type A and Type B CCK receptor antagonists were chosen based on our previously published observations (24,25). L-364,718 was given in doses of 0.1-1 mg/kg and L-365,260 was given in doses of 12.5-25 mg/kg. Both receptor antagonists were given intraperitoneally 30 minutes prior to treatment with a CCK secretagogue, while controls received an equal volume of vehicle (dimethyl sulfoxide, Tween-80, and saline: 1:1:8;v/v/v). Following a 30-minute treatment with orogastric saline, oleate or SBTI, gastric mucosal injury was induced with acidified ethanol and macroscopic injury determined as previously described. In the L-364,718 experiment, the effect of this antagonist against CCK-8 (5 nmol/kg iv) was examined as a positive control (24). In the L-365,260 experiment, the effect of this antagonist against gastrin-17 (25 pmol/kg iv) was assessed as a positive control (25).

Effects of Type A CCK Receptor Blockade on Mild Irritant Induced Gastric Injury

Although the Type A CCK receptor antagonist, L-364,718 did not exacerbate gastric injury from acidified ethanol (see Results), there was a trend toward increased gastric injury. Because acidified ethanol causes extensive macroscopic damage, additional studies were undertaken with L-364,718 to assess its effect on gastric injury from luminal irritants that result in mild or superficial injury to the gastric mucosa. This was accomplished with an anesthetized model of gastric injury (22,34). Accordingly, rats were given either intraperitoneal vehicle or L-364,718 (0.1-1 mg/kg) for 30 minutes. After 30 minutes, all rats (n = 5 per group) were anesthetized with xylazine and ketamine. A mid-line laparotomy was performed, the gastroesophageal junction and pylorus were ligated, and the gastric contents were aspirated. After aspiration, 3 ml of either the bile acid 5 mM acidified taurocholate or 20 % ethanol was introduced into the gastric lumen to induce gastric injury, while controls received intraluminal water. Bile acids such as
taurocholate are found in bile in concentrations ranging from 1-5 mM (31). The concentration of ethanol represents that of a 40 proof alcohol containing cocktail. Thus, both irritants have clinical significance. Because this model of gastric injury has been shown to result in gastric injury 10 minutes after introduction of luminal irritant, rats were killed 10 minutes after receiving either injurious agent, and macroscopic injury determined (22,34).

We also examined whether endogenous CCK mediates adaptive cytoprotection with the mild irritant 5 mM acidified taurocholate because bile acids also elicit the release of CCK. Thus, rats were given a 1 ml orogastric bolus of either 5 mM acidified taurocholate or water for 30 minutes prior to exposing the stomach to acidified ethanol for 5 minutes. This latter experiment (n = 5/group) was done in the presence and in the absence of Type A CCK receptor blockade.

_Assessment of NOS in CCK Secretagogue-Induced Gastroprotection and Gastric Hyperemia_

**NOS Inhibitor Studies**

Because NOS inhibitors have been shown to inhibit exogenous CCK gastroprotection (16,33), the effects of CCK secretagogues on gastroprotection and GMBF were assessed in the presence and in the absence of NOS inhibition. For the injury studies, conscious rats were given subcutaneous saline, the non-selective NOS inhibitor, N\(^{G}\)-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg), or the selective NOS-2 inhibitor, aminoguanidine (45 mg/kg) 15 minutes prior to a 30-minute treatment with 1 ml of orogastric saline, SBTI, or oleate. After 30-minutes, gastric injury was induced with acidified ethanol and macroscopic injury determined.

The effects of L-NAME and aminoguanidine on changes in GMBF were similarly assessed without exposing the stomachs to a luminal irritant using the laser Doppler method described
above. In addition, the effects of L-NAME on SBTI-induced gastroprotection and hyperemia were assessed at 10 minutes after administration of SBTI as this was the earliest time point for hyperemia. The reversibility of L-NAME was also examined by giving L- or D- arginine (300 mg/kg ip) concurrently with L-NAME in both the gastroprotection and GMBF studies (15). We have previously shown that this dose of aminoguanidine effectively inhibits gastric NOS-2 (12,22). A sample size of 5 or more rats per group was used in all NOS inhibitor studies.

**Protein Extraction and Western Immunoblot Analysis for NOS Isoforms**

Because Brzozowski *et al.* have previously demonstrated that CCK is associated with increased NOS-2 mRNA in rat gastric mucosa (13), we performed Western immunoblot analysis to estimate and compare the content of all 3 NOS isoforms in the stomach after treatment with saline, CCK-8, oleate, or SBTI. Following sacrifice of rats in which gastric mucosal NOS analysis was to be determined, each stomach was rapidly removed and mucosal homogenates were prepared. Tissue samples were immediately frozen in liquid nitrogen until protein extraction and Western immunoblot analysis for NOS-1 (neural), NOS-2 (inducible), and NOS-3 (endothelial) (12). To estimate and compare the content of gastric NOS isoform, each gastric mucosal sample was added to 1 ml of lysis buffer (10 mM Tris, pH 8.6, fluoride, 1.5 mM MgCl₂, 0.5% NP-40, 100 µM phenylmethanesulfonfyl, 100 µg/ml aprotinin, and 10 µg/ml leupeptin) and then subjected to two 30-second bursts of a Polytron (Vertishear). These samples were then transferred to microfuge tubes and centrifuged for 10 minutes at 11,000 g. The supernatant was removed and added to sample buffer (125 mM Tris, pH 6.8, 2% SDS, 5% glycerol, 1% β-mercaptoethanol, and 0.003% bromphenol blue). Protein concentrations within each homogenate were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) prior to the addition of sample buffer. Proteins were separated by SDS (7.5%)-
PAGE, using 40 μg of protein per sample in iNOS determinations and 80 μg of protein per sample in eNOS and nNOS determinations. Resultant proteins were electroblotted onto nitrocellulose membranes and incubated for 1 hour at room temperature in blocking solution (5% nonfat dried milk, 0.1% Tween 20, and PBS). The resultant blot was then washed twice in 0.1% Tween 20-PBS followed by a 1-hour incubation with either a specific polyclonal anti-iNOS antibody (1:2,000 dilution) or a specific polyclonal anti-eNOS or anti-nNOS antibody (1:700 dilution). Blots were then washed twice and incubated with a horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin as a secondary antibody (1:10,000 dilution for iNOS and 1:5,000 for eNOS or nNOS) for 1 hour. After two final washes, the immune complexes were visualized with the use of enhanced chemiluminescence detection (Amersham; Arlington Heights, IL). The autoradiograph was then assessed semiquantitatively by computer-assisted densitometry and reported as mean ± SE of relative densitometric units.

**Gastric Mucosal NOS Activity**

Gastric mucosal NOS activity was assayed by following the conversion of L-[H³] arginine to L-[H³] citrulline as described by Brown *et al.* (2), with some modifications. Rats (n = 5/group) were given saline, oleate (100 mM), SBTI (100 mg), or CCK-8 (5 nmol/kg, iv) for 30 minutes then killed without exposure to a luminal irritant. Stomachs were excised and opened along the greater curvature, the mucosa was scraped away from the underlying muscle and serosa, and the tissue snap frozen in liquid nitrogen. For crude homogenate NOS activity, mucosal tissue was homogenized in homogenization buffer (3 μl/μg; pH 7.4) containing 10 mM Hepes, 320 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, soybean trypsin inhibitor (30 μg/ml) and Sigma mammalian protease inhibitor cocktail (P8340; 45 μg/ml). Samples were homogenized at 4° C three times at 10 seconds using a Polytron homogenizer, followed by sonication three times at 10
seconds to break cellular membranes. Homogenized tissue was then centrifuged at 4°C for 30 min at 10,000g, and the supernatant was saved for NOS activity. For membranous protein extraction, gastric mucosal tissue was homogenized as above and the supernatant sonicated three times, 10 seconds each, on ice and then centrifuged again at 4°C for 1 hour at 100,000g. The supernatant was discarded and the pellet was washed in 2 mL of homogenization buffer and then centrifuged again at 100,000g for 30 minutes at 4°C. The pellet was resuspended in 400 µl of cold homogenization buffer containing 10% (v/v) glycerol.

For both crude homogenates and membranous protein fractions, 20 µl of supernatant was then incubated for 15 minutes at 37°C in various incubation solutions containing 50 µl of either assay buffer alone or with 5 mM EGTA, a calcium chelator, or the non-selective NOS inhibitor N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA; 2 mM). The assay buffer contained 1 mM NADPH, 40 mM KH\textsubscript{2}PO\textsubscript{4}, 150 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 5 mM L-valine, 5 mM L-arginine, and 1 µCi [H\textsuperscript{3}]-arginine per sample (pH 7.4). Because isolation of the membranous protein fraction removes necessary NOS co-factors, the assay buffer for the membranous protein fraction contained the above with the addition of 2 µM flavin adenine dinucleotide, 15 µM 6R-tetrahydrobiopterin, and 2 units calmodulin per sample. After incubation, the enzymatic reaction was terminated by placing samples in an ice bath and immediately adding 200 µl of an ice-cold 1:1 suspension of Dowex 50W X-8, a cation-exchange resin (Biorad; Hercules, Ca; pH 5.5). The resin was briefly centrifuged and 50 µl of supernatant was removed for estimation of L- [H\textsuperscript{3}] citrulline formation by liquid scintillation counting. Total NOS activity was the product formed that was inhibited by 2 mM L-NMMA. Citrulline formation that was inhibited by 2 mM L-NMMA, but not 5 mM EGTA was used to define calcium-independent, or inducible NOS activity. Constitutive, or calcium-dependent, activity was the difference between total and calcium-independent activity.
Data are expressed as micromoles of \([\text{H}^3]\) citrulline formed ± the SE for each experimental group. Protein determinations of supernatant were conducted using the BCA assay.

**Chemicals**

Nitrocellulose filters were purchased from Schleicher Schuell (Keene, NH), and X-ray film (T-MAT) was purchased from Eastman Kodak (Rochester, NY). The Enhanced Chemiluminescence System for Western immunoblot analysis was made by Amersham (Arlington Heights, IL). The BCA protein assay was from Pierce. The polyclonal antibody against NOS-2 was developed by the Trauma Research Center at the University of Texas Medical School at Houston, Texas. The efficacy of this antibody probe has been previously published (22). The polyclonal antibodies against NOS-1 and NOS-3 were obtained from Transduction Laboratory (Lexington, KY). Both L-364,718 (also known as MK-329) and L-365,260 were the generous gifts of Dr. Roger Friedinger from Merck Research Laboratories in West Point, Pennsylvania. Each antagonist was dissolved in 1:1 dimethylsulfoxide (DMSO) – Tween-80. This solution was subsequently diluted with 0.9% saline to a final concentration of 10% DMSO and 10% Tween-80. L – [\text{H}^3] arginine was obtained from Amersham Pharmacia Biotech (Piskataway, New Jersey). All other reagents, including gastrin, CCK, oleate, SBTI, L-NAME, taurocholate, and ethanol were of molecular biology grade and were purchased from Sigma Chemical (St. Louis, MO). Aminoguanidine was dissolved in 0.1 N hydrochloric acid and subsequently neutralized (pH 7.4) with 0.1 N sodium hydroxide.

**Statistics**

All values in the figures and text are expressed as mean ± SE of \(n\) observations, where \(n\) is the number of animals examined. Statistical significance was determined using analysis of variance
followed by Scheffé post hoc test analysis or by an unpaired Student’s T-test. \( P < 0.05 \) was considered to be statistically significant.

RESULTS

*CCK Secretagogues Prevent Gastric Injury from Acid, Base, and Ethanol*

The effects of transduodenal and orogastric CCK secretagogues against gastric injury caused by acidified ethanol are shown in Table 1. As demonstrated, both transduodenal and orogastric administration of CCK secretagogues were effective at preventing acidified ethanol-induced macroscopic gastric injury when compared to rats receiving saline. There was no significant difference in the magnitude of gastroprotection observed between orogastric or transduodenal administration of the CCK secretagogues. Both orogastric oleate and SBTI were effective in preventing the extent of macroscopic damage caused by longer periods of exposure to acidified ethanol (i.e. 1 hour) when compared with saline treated animals (21 ± 6\(^*\), 18 ± 7\(^*\) vs. 118 ± 21 mm\(^2\); \(*p< 0.001\)). Furthermore, SBTI-induced gastroprotection was present as early as 10 minutes after its administration (26 ± 5 mm\(^2\)). Table 1 also shows that both CCK secretagogues were efficacious at preventing gastric injury caused by concentrated acid or concentrated base.

Results of light microscopic evaluation of saline and oleate treated animals were identical to those previously published with exogenous CCK and gastrin (24,25). As shown in Figure 1, rats treated with saline followed by acidified ethanol had obliteration of the surface epithelium and loss of the mucosa in many areas down through the gastric pits with disruption of the glandular architecture. In addition, the interstitium was often edematous, and frank hemorrhage was frequently observed. The preponderance of mucosal injury in the saline treated controls consisted of primarily Type III injury (70 ± 7\%) and a smaller amount of deep Type IV injury.
(24 ± 8%). In comparison, oleate treated animals exposed to the same luminal irritant had significantly less morphologic injury to the gastric epithelium (Figure 1). The majority of the injury was confined to surface epithelial cells with minimal injury to gastric pits. The glandular architecture was also maintained. This experimental group had a much larger amount of Type II injury (47 ± 8%) and only a small amount of Type III injury (30 ± 7%). Roughly 5% (6 ± 4%) of the gastric epithelium was assessed as normal. Thus, CCK secretagogues provided both macroscopic and morphologic gastroprotection that was rapid in onset occurring as early as 10 minutes.

CCK Secretagogues Increase GMBF

The effects of oleate, SBTI, and CCK on GMBF over time are shown in Figure 2. As depicted, exogenous CCK and CCK secretagogues caused a significant increase in GMBF when compared to rats receiving saline. This hyperemic effect was rapid in onset, reaching significance at 10 minutes, peaking around 30 minutes, and returning to baseline values after 60 minutes. SBTI caused the most significant increases in GMBF at time points between 25 – 45 minutes that were also greater than that achieved with oleate or CCK (p < 0.03). Thus, this experiment demonstrated that CCK secretagogues augment GMBF and that this increase is present at the time the stomach is challenged with a luminal irritant.

Measurements of GMBF before and after exposure to the luminal irritant acidified ethanol are shown in Table 2. As shown, SBTI increased GMBF from baseline values when compared to saline treated rats in the absence of acidified ethanol. Following exposure of the gastric mucosa to acidified ethanol, GMBF increased in control rats. There was no further increase in GMBF in
SBTI treated rats with exposure to the luminal irritant, and blood flow remained significantly increased when compared to saline treated rats.

*Orogastric Oleate and SBTI Increase Plasma CCK Levels*

The results of the CCK RIA determinations are shown in Table 3. Both orogastric oleate and SBTI significantly increased plasma CCK levels 30 minutes after their administration when compared to these levels in animals receiving orogastric saline. The rise in plasma CCK levels was of similar magnitude to that obtained with a gastroprotective dose of CCK, given for 10 minutes, suggesting that the gastroprotective effect of CCK has physiological significance.

*Type A CCK Receptor Blockade Negates CCK Secretagogue Induced Gastroprotection*

As shown in Figure 3, the Type A CCK receptor antagonist, L-364,718, at a dose of 1 mg/kg, almost completely abolished the gastroprotective effects of orogastric oleate and SBTI. The lower dose of L-364,718 (0.1 mg/kg) partially reversed oleate and SBTI-induced gastroprotection (66 ± 7 vs. 34 ± 8 and 77 ± 10 vs. 33 ± 9 mm$^2$; p < 0.01). Type A CCK receptor blockade did not significantly increase gastric injury from acidified ethanol. L-364,718 was also effective at preventing exogenous CCK-induced gastroprotection (94 ± 14 vs. 6 ± 3 mm$^2$; p < 0.001) as previously reported (24). The Type B CCK receptor antagonist, L-365,260 did not have any inhibitory effects on the ability of CCK secretagogues to prevent acidified ethanol-induced gastric injury when compared to the vehicle/CCK secretagogue treated animals (Figure 4). However, L-365,260 did prevent the gastroprotective actions of exogenous gastrin-17. Taken together, the results of the Type A and Type B CCK receptor antagonist studies indicated that Type A CCK receptors are primarily responsible for mediating the gastroprotective actions of CCK and CCK secretagogues.
Type A CCK Receptor Blockade Exacerbates Gastric Injury from Bile and Ethanol

The effects of L-364,718 on bile acid and 20% ethanol-induced gastric injury are shown in Figure 5. In the absence of L-364,718, both acidified taurocholate and 20% ethanol caused minimal macroscopic gastric injury as previously reported (5,22). However, these same mild irritants caused significantly more macroscopic damage to the gastric mucosa in the presence of Type A CCK receptor blockade. Moreover, the lower dose of L-364,718 also exacerbated gastric injury from these luminal irritants \((6 \pm 3 \text{ vs. } 1.8 \pm 0.4 \text{ and } 7.5 \pm 4 \text{ vs. } 2.3 \pm 0.4 \text{ mm}^2; p < 0.01\), suggesting that endogenous CCK plays a role in maintaining mucosal integrity. L-364,718 alone did not cause any macroscopic injury to the stomachs when given to rats followed by intragastric water (i.e. no luminal irritant given).

Endogenous CCK Does Not Contribute to Adaptive Cytoprotection

Table 4 shows the effects of Type A CCK receptor blockade on 5 mM acidified taurocholate-induced adaptive cytoprotection. As shown, in the absence of L-364,718, exposure of the stomach to the mild irritant acidified taurocholate prevented gastric injury from acidified ethanol when compared to control rats, consistent with adaptive cytoprotection. However, while L-364,718 attenuated CCK and CCK secretagogue induced gastroprotection (Figure 3), Type A CCK receptor blockade failed to reverse or diminish mild irritant induced adaptive cytoprotection. These data suggest that endogenous CCK does not play a role in the phenomenon of adaptive cytoprotection.

NOS Inhibition Negates CCK Secretagogue-Induced Gastroprotection and Gastric Hyperemia
The effects of oleate and SBTI on gastroprotection and GMBF in the presence and in the absence of NOS inhibition with L-NAME are illustrated in Figure 6. As shown, in the absence of L-NAME, both oleate and SBTI significantly increased GMBF and resulted in gastroprotection when compared to saline treated rats. Administration of the non-selective NOS inhibitor, L-NAME, negated the gastroprotective actions of oleate and SBTI and prevented the gastric hyperemic response induced by these two CCK secretagogues. L-NAME also significantly reduced GMBF in rats receiving saline and exacerbated acidified ethanol-induced gastric injury. Administration of L-arginine, but not D-arginine, reversed the effects of L-NAME on CCK secretagogue-induced gastroprotection and GMBF (not shown). L-NAME also blocked SBTI-induced gastric protection and hyperemia following a 10-minute treatment with SBTI, a time point where gastroprotection and hyperemia just became significantly increased (not shown). These results were not significantly different from those in Figure 6. In contrast, selective NOS-2 inhibition with aminoguanidine failed to reverse or attenuate CCK-induced gastroprotection or hyperemia (Table 5). However, this dose of aminoguanidine effectively prevented LPS-induced (20 mg/kg ip) increases in gastric luminal fluid accumulation of nitrates and nitrites (Griess reaction) as previously reported (not shown) (12). Taken collectively, these experiments suggested that CCK secretagogue-induced gastroprotection and gastric hyperemia are mediated by nitric oxide produced primarily from the constitutive NOS isoforms, NOS-1 and NOS-3, and not from the inducible isoform, NOS-2.

*Exogenous CCK and CCK Secretagogues Increase NOS activity but not NOS Isoform Immunoreactivity*

The effects of exogenous CCK and CCK secretagogues on NOS isoform immunoreactivity are shown in Figure 7 and Table 6. As demonstrated, neither exogenous CCK, nor the CCK
secretagogues, oleate and SBTI, increased gastric NOS-1, NOS-2, or NOS-3 isoform immunoreactivity when compared to saline treated rats. NOS activity determinations from membranous fractions (membrane bound proteins, i.e. NOS-3) and crude homogenates (cytosolic and membrane bound proteins, i.e NOS 1 and 2) are shown in Figures 8 and 9. As shown in Figure 8, CCK and CCK secretagogues increased calcium-dependent NOS activity when compared to rats receiving saline. In contrast, no change in calcium-independent NOS activity (NOS-2) was detected in the crude homogenates of gastric mucosa obtained from the same treatment groups. Similarly, there were no significant differences in crude homogenate calcium-dependent NOS activity (NOS-1) observed in the identical treatment groups (data not shown). Taken together, the NOS studies indicated that exogenous CCK and CCK secretagogues increase NOS activity through changes in the activity of the enzyme and not via increases in NOS isoform protein. Moreover, this increase is due to changes in activity of membrane-bound NOS as opposed to NOS contained within the cytosol.

DISCUSSION

This study demonstrated that oleate and SBTI are potent gastroprotective agents against a variety of luminal irritants according to macroscopic and morphologic criteria. These protective actions are mediated by activation of Type A CCK receptors. Furthermore, both oleate and SBTI caused a significant increase in plasma CCK levels that were similar in magnitude to those achieved with a gastroprotective dose of exogenous CCK. These findings suggest that another physiologic action for CCK exists. In addition to its other well-described actions on gallbladder contraction and pancreatic protein secretion, this study suggests that release of endogenous CCK in response to a meal also enables the stomach to withstand injury from ingested irritants such as ethanol or from reflux of bile. Moreover, the fact that Type A CCK receptor blockade exacerbated gastric
injury from 20% ethanol and the bile acid 5 mM acidified taurocholate further suggests that endogenous CCK plays an important role in the intrinsic gastric mucosal defense system. However, the finding that Type A CCK receptor blockade failed to reverse or attenuate adaptive cytoprotection indicates that CCK does not play a role in this process.

This study also demonstrated that CCK secretagogues, like exogenous CCK, augment gastric mucosal blood flow. Although, Konturek et al. and Heinemann et al. demonstrated that exogenous CCK-induced hyperemia is reversed by non-selective NOS inhibition (11,16), the effect of NOS inhibition on CCK secretagogues was not examined. We found that gastric hyperemia in response to CCK secretagogues was also negated by non-selective NOS inhibition with L-NAME. The effects of L-NAME were in turn overcome with excess substrate for NOS because L-arginine, but not D-arginine reversed the effects of L-NAME. In addition, L-NAME prevented oleate and SBTI-induced gastroprotection, effects that were likewise reversed by L-arginine, but not D-arginine. These findings with L- and D-arginine suggest that the effects of L-NAME on CCK secretagogue-induced gastric hyperemia and gastroprotection are due to inhibition of nitric oxide production by constitutive isoforms of NOS and not due to any non-specific effects of L-NAME. In contrast to L-NAME, aminoguanidine, a selective inhibitor of NOS-2, failed to reverse CCK-induced gastric hyperemia or CCK-induced gastroprotection. Taken together, these NOS inhibitor studies indicated that the gastroprotective and hyperemic actions of CCK are mediated by increased nitric oxide production from primarily the constitutively expressed NOS isoforms, NOS-1 and NOS-3.

The NOS isoforms are all homodimeric bifunctional enzymes consisting of a reductase domain, with NADPH, FAD, and FMN binding sites, and an oxygenase domain with binding sites for substrate, heme, and tetrahydrobiopterin (10). Unlike gastric NOS-1 (neural) and NOS-2
(inducible), which are found in the cytosol, gastric NOS-3 (endothelial) is primarily (>90%) membrane-bound because of myristoylation of the gly2 residue (9,21). In the gastric mucosa, there is considerably more constitutive or calcium-dependent NOS activity relative to many other tissues (12,32). Nitric oxide produced in the gastric mucosa from the constitutively expressed NOS isoforms plays an integral part in maintaining mucosal integrity, regulating gastric mucosal blood flow, and in cell signaling between neurons (1). Interestingly, only 10% of calcium-dependent NOS activity in gastric mucosa is from the particulate or membranous fraction because over 90% of calcium-dependent NOS activity in the gastric mucosa is generated from NOS proteins found in the cytosol (29). This implies that neuronal NOS or NOS-1 is primarily responsible for the generation of NO by the constitutive NOS isoforms in the gastric mucosa. However, gastric hyperemia in response to nitric oxide is believed to be derived principally from nitric oxide generated by endothelial NOS or NOS-3 because it is associated with mucosal blood vessels (30). In contrast, NOS-1 is localized to surface epithelial cells and is believed to be involved in cell signaling (29). Thus, it is noteworthy that in the gastric mucosa approximately 90% of NOS-3 resides within the membranous fraction of protein homogenates and only about 10% resides within the cytosol. Consequently, NOS activity assays of the crude homogenate, which contains both cytosolic and membrane bound proteins, will reflect changes in NOS-1 and NOS-2, but not NOS-3 because of the relatively small contribution NOS-3 makes to overall NOS activity in the cytosol.

In our study, we did not find any significant change in calcium-dependent or independent NOS activity in the crude homogenate. This suggests that CCK or CCK secretagogues did not significantly influence NOS-1 and NOS-2 activity as these isoforms are primarily cytosolic proteins in the stomach and any changes in their activity should have been detected in this
sample. In contrast, when the membranous fraction was examined, there was a significant increase in calcium-dependent NOS activity suggesting that NOS-3 activity increased following CCK and CCK secretagogues. L-NAME not only prevented CCK secretagogue-induced gastroprotection, it also blunted the hyperemic response, further suggesting that NOS-3 is primarily involved because NOS-3 plays an important role in gastric mucosal blood flow (30). Thus, we believe our results indicate that exogenous CCK and CCK secretagogues increase activity of NOS-3 that causes a resultant increase in gastric mucosal blood flow, preparing the gastric mucosa to withstand injury from a damaging luminal insult.

Our observations differ from those made by Brzozowski et al. They found that CCK given intraperitoneally or intracerebrally resulted in increased NOS-2 or iNOS mRNA in the gastric mucosa (13). Constitutive NOS (NOS-1 or NOS-3 not specified) mRNA did not change. NOS-2 protein was not measured, NOS-3 and NOS-1 were not specifically examined, and NOS activity assays were not undertaken. However, gastric luminal accumulation of nitrates and nitrites increased following CCK, and this effect was abolished by L-NAME. The effect of selective NOS-2 inhibition was not assessed. From these results, they concluded that CCK-induced gastroprotection was due to increased nitric oxide production from upregulation of iNOS or NOS-2. In contrast, we found no changes in NOS isoform immunoreactivity and that aminoguanidine failed to reverse CCK-induced gastroprotection and hyperemia. Furthermore, aminoguanidine prevented LPS-induced accumulation of gastric luminal nitrates and nitrites whereas L-NAME did not, as previously reported (12).

To our knowledge, this is the first report to examine the effects of exogenous CCK and CCK secretagogues on gastric NOS isoform immunoreactivity and gastric mucosal NOS activity. We
found that exogenous CCK and CCK secretagogues increase constitutive or calcium-dependent NOS activity in the membranous fraction of gastric mucosal homogenates when compared to controls, thus confirming increased constitutive NOS isoform activity as the NOS inhibition studies with L-NAME suggested. Although we did not anticipate any change in NOS isoform immunoreactivity due to the short thirty minute treatment time, Western immunoblot analysis was performed on both crude homogenates containing cytosolic and membrane bound proteins as well as on isolated membranous fractions to further examine the finding that CCK increased NOS-2 mRNA as previously observed by Brzozowski et al. (13). Neither exogenous CCK nor the CCK secretagogues, oleate or SBTI, was found to significantly alter NOS-1, NOS-2, or NOS-3 isoform immunoreactivity in either of the fractions prepared from gastric mucosal homogenates. Thus, our Western immunoblot analysis of gastric NOS isoforms indicated that the increased NOS activity measured in gastric mucosa from CCK and CCK secretagogue-treated rats was due to changes in activity of the NOS enzymes, and not due to changes in NOS protein. We also think that our findings with L-NAME are consistent with those of Brzozowski et al. While L-NAME abolished CCK-induced accumulation of nitrates and nitrites within the gastric lumen in their study (13), it is noteworthy that L-NAME preferentially inhibits the constitutive NOS isoforms as opposed to the inducible isoform (28). As a result, we would interpret their data differently and suggest that the nitrates and nitrites that accumulated within the gastric lumen were derived from either NOS-1 or NOS-3 and not NOS-2. Moreover, the fact that aminoguanidine, which has a 50-fold difference in affinity for the mouse NOS-2 isoform versus NOS-1 and a 500-fold difference versus NOS-3 (35), failed to reverse CCK-induced gastroprotection or hyperemia further suggests that NOS-2 does not play a role. Taken together, these findings dispute the notion that NOS-2 mediates CCK-induced gastroprotection and strongly suggest that constitutive NOS isoforms are responsible for this effect.
In conclusion, this study demonstrated that CCK secretagogues augment gastric mucosal blood flow and are potent gastroprotective agents against a variety of luminal irritants. Type A CCK receptor blockade negated CCK secretagogue-induced gastroprotection and exacerbated gastric injury from bile and ethanol, but did not block adaptive cytoprotection. The plasma CCK levels achieved with CCK secretagogue administration were similar in magnitude to that achieved with a gastroprotective dose of exogenous CCK. These findings suggest that CCK is released in response to a meal and plays an important role in gastric mucosal defense against injury from ingested ethanol or duodenal gastric reflux of bile. The mediator responsible for CCK-induced gastroprotection appears to be nitric oxide because inhibition of nitric oxide synthase negated CCK secretagogue-induced gastroprotection and hyperemia, effects that were reversed by L-arginine but not D-arginine. Moreover, exogenous CCK and CCK secretagogues increased calcium-dependent NOS activity, suggesting that nitric oxide produced by the constitutive NOS isoforms is primarily responsible for the protective and hyperemic actions associated with CCK. Because the increase in calcium-dependent NOS activity was found in the membranous fraction, which consists primarily of NOS-3, we speculate that this increase in NOS activity and the resultant increase in blood flow are principally due to the effects of CCK on NOS-3 and not NOS-1 or NOS-2.
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REFERENCES


FIGURE LEGENDS

Figure 1  (Top Panel) Light microscopy of rat stomach treated with orogastric saline for 30 minutes followed by exposure to acidified ethanol for 5 minutes shows typical areas of Type III injury, characterized by obliteration of surface epithelial cells, loss of gastric pit cells, and damage to the upper gastric glands (x 250).  (Bottom Panel) Light microscopy of rat stomach treated with orogastric oleate for 30 minutes followed by exposure to acidified ethanol for 5 minutes results in gastric mucosal protection, with only minor disruption of the superficial epithelium and gastric pits. The glands are intact (x 250).

Figure 2  Effects of transduodenal saline, oleate (100 mM), or SBTI (100 mg) and intravenous CCK (5 nmol/kg) on gastric mucosal blood flow over time in the absence of a luminal irritant. Blood flow was recorded continuously with laser Doppler after administration of each substance, and is reported as the average of the peak blood flow in each rat. Values are mean ± SE; n = 5 for all groups. * P < 0.05 vs. CCK, oleate, and SBTI.

Figure 3  Effect of intraperitoneal Type A cholecystokinin receptor antagonist L-364,718 given 30 minutes before a 30-minute orogastric treatment with saline, oleate, or SBTI on macroscopic gastric injury from acidified ethanol. Values are mean ± SE; n = 5 for all groups. *P < 0.001 vs. vehicle/saline. **P < 0.002 vs. vehicle/oleate or vehicle/SBTI.
Figure 4  Effect of intraperitoneal Type B cholecystokinin receptor antagonist L-365,260 given 30 minutes before a 30-minute orogastric treatment with saline, oleate (100mM), or SBTI (100mg) on macroscopic gastric injury from acidified ethanol. Gastrin-17 (25 pmol/kg) was given intravenously 10 minutes before acidified ethanol. Values are mean ± SE; n = 5 for all groups. *P < 0.001 vs. saline counterpart. +P < 0.001 vs. vehicle/gastrin-17.

Figure 5  Effect of intraperitoneal Type A cholecystokinin receptor antagonist L-364,718 or vehicle on rat macroscopic gastric injury caused by exposure of the stomach to 3 mL of 5 mM acidified taurocholate (ATC) or 20% ethanol while control stomachs were exposed to distilled water. L-364,718 was given 30 minutes prior to exposing the stomach to an irritant or water. Data are mean ± SE; n = 5 for all groups. *P < 0.01 vs. vehicle counterpart.

Figure 6  Effect of N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) on CCK secretagogue-induced gastroprotection and gastric hyperemia. Saline or L-NAME (10mg/kg) given subcutaneously 15 minutes prior to a 30-minute treatment with 1 ml of saline, oleate (100 mM) or SBTI (100 mg). Gastric injury caused by acidified ethanol (150 mM HCl/50% ethanol, 1ml orogastrically) given 5 minutes after indicated treatment is depicted by bar graph. GMBF determined in uninjured stomachs with laser Doppler for 30 minutes after 1 ml transduodenal injection of saline, oleate or SBTI is depicted by line graph. * P < 0.005 vs. saline/saline. ** P< 0.01 vs. saline/oleate or saline/SBTI.
Figure 7  Representative Western immunoblot analyses for neural (NOS-1), inducible (NOS-2), and endothelial (NOS-3) NOS in rat gastric mucosal membranous fractions from 3 different immunoblots after a 30-minute treatment with saline, CCK, oleate, or SBTI. Stomachs were not exposed to a luminal irritant. Each lane was loaded with 80 μg of protein. A positive control for each NOS isoform was loaded in the far right lane of each blot.

Figure 8  Effects of saline, CCK, SBTI and oleate on calcium-dependent NOS activity in the membranous fraction from gastric mucosal homogenates taken from rats receiving saline, CCK (5 nmol/kg), oleate (100 mM), or SBTI (100 mg) for 30 minutes without exposure of the stomach to a luminal irritant. NOS activity is reported as mean ± SE; n = 5 /group. *P < 0.05 for all groups vs. saline.

Figure 9  Effects of saline, CCK, SBTI and oleate on calcium-independent NOS activity in the crude homogenate from gastric mucosal homogenates taken from rats receiving saline, CCK (5 nmol/kg), oleate (100 mM), or SBTI (100 mg) for 30 minutes without exposure of the stomach to a luminal irritant. NOS activity is reported as mean ± SE; n = 5/group. P = NS.
Table 1  Effects of CCK Secretagogues against Gastric Injury in the Conscious Rat

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>INJURY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transduodenal</td>
<td>150 mM HCl/50% Ethanol (mm²)</td>
</tr>
<tr>
<td>Saline</td>
<td>118 ± 22</td>
</tr>
<tr>
<td>Oleate (100 mM)</td>
<td>35 ± 7*</td>
</tr>
<tr>
<td>SBTI (100 mg)</td>
<td>31 ± 10*</td>
</tr>
<tr>
<td>Orogastric</td>
<td>150 mM HCl/50% Ethanol (mm²)</td>
</tr>
<tr>
<td>Saline</td>
<td>123 ± 20</td>
</tr>
<tr>
<td>Oleate (100 mM)</td>
<td>34 ± 8*</td>
</tr>
<tr>
<td>SBTI (100 mg)</td>
<td>33 ± 9*</td>
</tr>
<tr>
<td>Orogastric</td>
<td>0.2N NaOH (mm²)</td>
</tr>
<tr>
<td>Saline</td>
<td>156 ± 25</td>
</tr>
<tr>
<td>Oleate (100 mM)</td>
<td>51 ± 12*</td>
</tr>
<tr>
<td>SBTI (100 mg)</td>
<td>44 ± 11*</td>
</tr>
<tr>
<td>Orogastric</td>
<td>0.75N HCl (mm²)</td>
</tr>
<tr>
<td>Saline</td>
<td>150 ± 17</td>
</tr>
<tr>
<td>Oleate (100 mM)</td>
<td>38 ± 10*</td>
</tr>
<tr>
<td>SBTI (100 mg)</td>
<td>30 ± 9*</td>
</tr>
</tbody>
</table>

Transduodenal or orogastic saline, oleate and SBTI were given as a 1-ml bolus for 30 minutes prior to exposure to a 1-ml bolus of luminal irritants, acidified ethanol (AE), concentrated acid, or concentrated base. Macroscopic injury is reported as mean ± SE (n = 5/group); *P < 0.005 vs. saline counterpart.
Table 2  Effects of Soybean Trypsin Inhibitor on Gastric Mucosal Blood Flow before and after Exposure to Acidified ethanol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GMBF (% baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Irritant</td>
</tr>
<tr>
<td>Saline</td>
<td>87.9 ± 9</td>
</tr>
<tr>
<td>SBTI (100 mg)</td>
<td>217.3 ± 30.9*</td>
</tr>
</tbody>
</table>

Blood flow was recorded continuously with laser Doppler in anesthetized model. After baseline was determined, transduodenal saline or SBTI (100 mg) was given and blood flow recorded for 30 minutes. Acidified ethanol (150 mM HCl/50% ethanol) was administered intragastrically, and blood flow was recorded for 5 minutes. Blood flow is reported as the average of the peak blood flow. Values are mean ± SE; n = 5 for all groups. *P < 0.05 vs. saline counterpart. ** P < 0.05 vs. saline before irritant.
Table 3  Effects of Saline, CCK, Oleate, or SBTI on Plasma CCK Levels in the Conscious Rat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma CCK (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (IV)</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>CCK (5 nmol/kg IV)</td>
<td>8.7 ± 0.9*</td>
</tr>
<tr>
<td>Saline (OG)</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>Oleate (100 mM OG)</td>
<td>9.6 ± 1.8*</td>
</tr>
<tr>
<td>SBTI (100 mg OG)</td>
<td>16.9 ± 2*</td>
</tr>
</tbody>
</table>

Intravenous saline or CCK was given for 10 minutes without exposing the stomach to a luminal irritant. Orogastric saline, oleate, or SBTI were given for 30 minutes without exposure of the stomach to a luminal irritant. Plasma CCK levels are reported as mean ± SE n = 5/group; * P < 0.008 vs. saline counterpart.
Table 4  Effect of Type A CCK-receptor Blockade on 5 mM Acidified taurocholate-induced Adaptive Gastroprotection from Acidified ethanol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Injury (mm²)</th>
<th>5 mM ATC</th>
<th>6 ± 3*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>115 ± 21</td>
<td>5 mM ATC</td>
<td></td>
</tr>
<tr>
<td>L-364,718 (1mg/kg)</td>
<td>134 ± 23</td>
<td></td>
<td>7 ± 5*</td>
</tr>
</tbody>
</table>

Type A CCK receptor antagonist L-364,718 given 30 minutes before a 30-minute treatment with water or 5 mM ATC on macroscopic gastric injury from acidified ethanol. Values are mean ± SE; n = 5 for all groups. *P < 0.001 vs. water counterpart.
Table 5  Effects of Aminoguanidine (AG) on CCK Induced Gastroprotection and Gastric Hyperemia in the Conscious Rat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Injury (mm²)</th>
<th>GMBF (% baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline/Saline</td>
<td>118 ± 22</td>
<td>100 ± 7.5</td>
</tr>
<tr>
<td>Saline/CCK</td>
<td>7 ± 3*</td>
<td>237 ± 10.8*</td>
</tr>
<tr>
<td>AG/Saline</td>
<td>115 ± 18</td>
<td>103 ± 7.8</td>
</tr>
<tr>
<td>AG/CCK</td>
<td>8 ± 4†</td>
<td>221 ± 9.6†</td>
</tr>
</tbody>
</table>

Values are mean ± SE; n = 5 rats/group. Saline or aminoguanidine (45 mg/kg, AG) was given intraperitoneally 15 minutes prior to a 30-minute treatment with subcutaneous saline or CCK (50 nmol/kg). In injury studies, acidified ethanol (150 mM HCl/50% ethanol, 1 ml orogastrically) was given for 5 minutes after indicated treatments. In GMBF studies, acidified ethanol was not given. GMBF was determined with laser Doppler for 30 minutes after administration of saline or CCK and is reported as average of the peak blood flow in each rat. *P < 0.001 vs. saline/saline. †P < 0.001 vs. AG/saline.
Table 6  Densitometric analysis of NOS-1, NOS-2 and NOS-3 immunoreactivity after 30-minute treatment with SBTI, oleate or CCK.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>iNOS</th>
<th>eNOS</th>
<th>nNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.372 ± 0.06</td>
<td>0.428 ± 0.01</td>
<td>0.360 ± 0.01</td>
</tr>
<tr>
<td>Oleate</td>
<td>0.495 ± 0.05</td>
<td>0.455 ± 0.04</td>
<td>0.318 ± 0.07</td>
</tr>
<tr>
<td>SBTI</td>
<td>0.437 ± 0.03</td>
<td>0.350 ± 0.11</td>
<td>0.363 ± 0.01</td>
</tr>
<tr>
<td>CCK</td>
<td>0.485 ± 0.02</td>
<td>0.479 ± 0.07</td>
<td>0.384 ± 0.01</td>
</tr>
</tbody>
</table>

Densitometry was performed of iNOS, eNOS and nNOS Western immunoblots (representative blots shown in Figure 7). Analysis for iNOS, eNOS and nNOS protein in gastric mucosal membranous fractions performed after 30-minute treatment with saline, CCK, oleate or SBTI. No significant differences were seen in any treatment groups compared with saline.
Figure 1
Figure 2
Figure 3
Figure 4
Macroscopic Gastric Injury (mm $^2$)

Figure 5
Figure 6
Figure 7
Membranous Calcium-Dependent NOS Activity

Figure 8
Crude Homogenate Calcium-Independent NOS Activity

Figure 9