Cholecystokinin secretagogue-induced gastroprotection: role of nitric oxide and blood flow

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We and others (7, 8, 16, 24, 26, 27, 33) have previously shown that exogenous CCK prevents macroscopic and morphological injury to the gastric mucosa caused by a variety of luminal irritants. The fact that gastroprotection was observed in doses approaching physiological 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. (16) later demonstrated that transduodenal oleate had gastroprotective actions against ethanol, and gastroprotection was negated by administration of a type A CCK receptor antagonist. 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 nonselective 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. (3) reported that CCK-induced gastroprotection is due to increased nitric oxide production from overexpression of inducible NOS (iNOS or NOS-2). 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 (30). 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 previously been published in abstract form (6, 23).

METHODS

Animals

Female Sprague-Dawley rats weighing ~200 g were used in all studies and were housed at constant room temperature with 12:12-h light-dark cycles. Rats were maintained on certified custom formula rat chow ordered in bulk to diminish the inherent variability in composition due to least-cost formulation that could alter basal gut peptide levels. Certified Teklad Rodent Diet (W) (formula 8728; Harlan Teklad, Madison, WI) contains 24% crude protein, 4% crude fat, and 4.5% crude fiber and provides 3.93 kcal/g gross energy. All experiments were performed in conscious rats deprived of food for 18–24 h 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 to 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 for the placement of transduodenal catheters. Nevertheless, the first set of experiments was performed by 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 elastomer, 0.030 in. ID; 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 postoperatively. Animals were allowed to recover for 7 days after the operation. After recovery, rats were fasted for 18–24 h and then given a 1-ml transduodenal bolus of oleate (100 mM), SBTI (100 mg), or saline for 30 min. This concentration of oleate and dose of SBTI have been shown to increase CCK levels in the rat (17, 20). After the 30-min 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 HCl results in macroscopic damage within 5 min, rats were killed 5 min 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 by using computerized planimetry. Damage was reported as square millimeters (means ± SE) for each experimental group. A sample size of seven 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-min treatment time, rats were given acidified ethanol for 5 min, and macroscopic injury was determined. 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 min. In additional rats, with the use of similar treatment groups, the exposure to acidified ethanol was extended to 1 h 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.

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

Morphological 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-min treatment with either orogastric saline or oleate. In these studies, rats were killed 5 min 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 (see acknowledgments). Sections (2 × 10 mm) of glandular mucosa were stained with hematoxylin and eosin and processed for routine microscopy by utilizing standard techniques. Gastric mucosal damage was assessed by previously published criteria, as follows: type I damage, involvement of luminal surface mucous cells only; type II damage, involvement of luminal surface and gastric pit mucous cells; type III damage, involvement of surface and gastric mucous cells as well as upper gland cells; type IV damage, severe injury to all surface and all or most of the 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 by a laser-Doppler method (25). Rats were
anesthetized with an intraperitoneal injection of xylazine and ketamine. After induction of anesthesia, the stomach was exposed by a midline incision. Through the nonglandular forestomach, a catheter was introduced to provide access for a Teflon-coated laser-optic flow probe (PeriFlux PF 409, 0.25-mm fiber separation; StandardProbe). The flow probe was positioned to allow contact with the glandular or acid-secreting portion of the stomach. After appropriate positioning of the probe was ensured, the stomach was allowed to equilibrate for 30 min. Following equilibration, mucosal blood flow to the stomach was recorded continuously with a laser-Doppler flow monitor (PeriFlux 4001 Masters; Perimed, Järfälla, Sweden). Blood flow was recorded for a 5-min period as the measurement of basal GMFB. Following basal GMFB determinations, a 1-ml injection of transduodenal saline, oleate (100 mM), or SBTI (100 mg) was given, and blood flow was recorded continuously for 60 min. In additional rats, exogenous CCK (5 nmol/kg iv) was given to compare the effects of CCK secretagogues against exogenous CCK-induced changes in GMFB. We also examined GMFB after the luminal irritant acidified ethanol in the presence and absence of CCK secretagogues. Although the type A CCK receptor antagonist L-364718 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-364718 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-364718 (0.1–1 mg/kg) for 30 min. After 30 min, all rats (n = 5/group) were anesthetized with xylazine and ketamine. A midline 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, whereas controls received intraluminal water. Bile acids such as taurocholate are found in bile in concentrations ranging from 1 to 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 min after introduction of luminal irritant, rats were killed 10 min after receiving either injurious agent, and macroscopic injury was 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 min before exposing the stomach to acidified ethanol for 5 min. 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 GMFB were assessed in the presence and in the absence of NOS inhibition. For the injury studies, conscious rats were given intravenous saline or the nonselective NOS inhibitor Nω-nitro-arginine methyl ester (LNAME; 10 mg/kg), or the selective NOS-2 inhibitor aminoguanidine (45 mg/kg) 15 min before a 30-min treatment with a CCK secretagogue, whereas controls received an equal volume of vehicle (dimethyl sulfoxide, Tween-80, and saline, 1:1:8, vol/vol/vol).

Following a 30-min treatment with orogastric saline, oleate, or SBTI, gastric mucosal injury was induced with acidified ethanol, and macroscopic injury was determined as in Assessment of CCK Secretagogues Against Gastric Injury from Luminal Irritants. In the L-364718 experiment, the effect of this antagonist against CCK-8 (5 nmol/kg iv) was examined as a positive control (24). In the L-365260 experiment, the effect of this antagonist against gastrin-17 (25 pmol/kg iv) was assessed as a positive control (25).
The effects of L-NAME and aminoguanidine on changes in GMBF were similarly assessed without exposing the stomachs to a luminal irritant by using the laser-Doppler method described in Assessment of CCK Secretagogues on GMBF. In addition, the effects of L-NAME on SBTI-induced gastroprotection and hyperemia were assessed at 10 min after administration of SBTI, because 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 five 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. (3) previously demonstrated that CCK is associated with increased NOS-2 mRNA in rat gastric mucosa, we performed Western immunoblot analysis to estimate and compare the content of all three NOS isoforms in the stomach after treatment with saline, CCK-8, oleate, or SBTI. After rats in which gastric mucosa was homogenized in homogenization buffer (3°C) for 30 min at 10,000 g, the supernatant was saved for NOS activity. For membranous protein extraction, gastric mucosal tissue was homogenized as above, the supernatant was sonicated three times, 10 s each, on ice, and then it was centrifuged again at 4°C for 1 h at 100,000 g. The supernatant was discarded, the pellet was washed in 2 ml of homogenization buffer, and then it was centrifuged again at 100,000 g for 30 min at 4°C. The pellet was resuspended in 400 μl of cold homogenization buffer containing 10% (vol/vol) glycerol.

For both crude homogenates and membranous fractions, 20 μl of supernatant was then incubated for 15 min with Merck Research Laboratories (West Point, PA). Each of either assay buffer alone or with 5 mM EGTA, a calcium chelator, or the nonselective NOS inhibitor N(G)-monomethyl-L-arginine (L-NMMA; 2 mM). The assay buffer contained 1 mM NADPH, 40 mM KH2PO4, 150 μM CaCl2, 1 mM MgCl2, 5 mM t-valine, 5 mM L-arginine, and 1 μCi l-[3H]arginine per sample (pH 7.4). Because isolation of the membranous protein fraction removes necessary NOS cofactors, the assay buffer for the membranous protein fraction contained the above with the addition of 2 μM FAD, 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 50WX-8, a cation-exchange resin (pH 5.5; Bio-Rad, Hercules, CA). The resin was briefly centrifuged, and 50 μl of supernatant was removed for estimation of [3H]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 [3H]citrulline formed (mean ± SE for each experimental group). Protein determinations of supernatant were conducted by 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 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-2 and NOS-3 were obtained from Transduction Laboratories (Lexington, KY). Both L-364674 (also known as MK-329) and L-365260 were the generous gifts of Dr. Roger Friedinger (Washington University School of Medicine, St. Louis, MO). Each antagonist was dissolved in 1:1 DMSO/Tween 80. This solution was subsequently diluted with 0.9% saline to a final concentration of 10% DMSO and 10% Tween 80. L-364674 was obtained from Promega (Madison, WI). All other reagents, including gastrin, CCK, and muscle and serosa, and the tissue was snap frozen in liquid nitrogen. For crude homogenate NOS activity, mucosal tissue was homogenized in homogenization buffer (3 μM CCK-8: pH 7.4) containing 10 mM HEPES, 320 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, 30 μg/ml SBTI, and Sigma mammalian protease inhibitor cocktail (P8340; 45 μg/ml). Samples were homogenized at 4°C three times for 10 s by using a Polytron homogenizer, followed by sonication three times for 10 s to break cellular membranes. Homogenized tissue was then centrifuged at 4°C for 30 min at 10,000 g, and the supernatant was saved for NOS activity. For membranous protein extraction, gastric mucosal tissue was homogenized as above, the supernatant was sonicated three times, 10 s each, on ice, and then it was centrifuged again at 4°C for 1 h at 100,000 g. The supernatant was discarded, the pellet was washed in 2 ml of homogenization buffer, and then it was centrifuged again at 100,000 g for 30 min at 4°C. The pellet was resuspended in 400 μl of cold homogenization buffer containing 10% (vol/vol) glycerol.

For both crude homogenates and membranous fractions, 20 μl of supernatant was then incubated for 15 min with Merck Research Laboratories (West Point, PA). Each of either assay buffer alone or with 5 mM EGTA, a calcium chelator, or the nonselective NOS inhibitor N(G)-monomethyl-L-arginine (L-NMMA; 2 mM). The assay buffer contained 1 mM NADPH, 40 mM KH2PO4, 150 μM CaCl2, 1 mM MgCl2, 5 mM t-valine, 5 mM L-arginine, and 1 μCi l-[3H]arginine per sample (pH 7.4). Because isolation of the membranous protein fraction removes necessary NOS cofactors, the assay buffer for the membranous protein fraction contained the above with the addition of 2 μM FAD, 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 50WX-8, a cation-exchange resin (pH 5.5; Bio-Rad, Hercules, CA). The resin was briefly centrifuged, and 50 μl of supernatant was removed for estimation of [3H]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 [3H]citrulline formed (mean ± SE for each experimental group). Protein determinations of supernatant were conducted by using the BCA assay.
oleate, SBTI, 1-NAME, taurocholate, and ethanol were of molecular biology grade and were purchased from Sigma (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 means ± SE of n observations, where n is the number of animals examined. Statistical significance was determined by 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 in preventing acidified ethanol-induced macroscopic gastric injury compared with 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 h) when compared with saline-treated animals (21 ± 6 and 18 ± 7 vs. 118 ± 21 mm², respectively; *P < 0.001). Furthermore, SBTI-induced gastroprotection was present as early as 10 min after its administration (26 ± 5 mm²). 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 with exogenous CCK and gastrin (24, 25). As shown in Fig. 1, rats treated with saline followed by acidified ethanol for 5 min show 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 (magnification, ×250). B: light microscopy of rat stomach treated with orogastric oleate for 30 min followed by exposure to acidified ethanol for 5 min results in gastric mucosal protection, with only minor disruption of the superficial epithelium and gastric pits. The glands are intact (magnification, ×250).

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>Ora gastric</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>Ora gastric</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>Ora gastric</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 orogastric saline, oleate, and soybean trypsin inhibitor (SBTI) were given as a 1-ml bolus for 30 min before exposure to a 1-ml bolus of luminal irritants, acidified ethanol, concentrated acid, or concentrated base. Macroscopic injury is reported as means ± SE (n = 5/group); *P < 0.005 vs. saline counterpart.
ponderance 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 morphological injury to the gastric epithelium (Fig. 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 morphological gastroprotection that was rapid in onset, occurring as early as 10 min.

**CCK Secretagogues Increase GMBF**

The effects of oleate, SBTI, and CCK on GMBF over time are shown in Fig. 2. As depicted, exogenous CCK and CCK secretagogues caused a significant increase in GMBF compared with rats receiving saline. This hyperemic effect was rapid in onset, reaching significance at 10 min, peaking around 30 min, and returning to baseline values after 60 min. SBTI caused the most significant increases in GMBF at time points between 25 and 45 min 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 that 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 3. Both orogastric oleate and SBTI significantly increased plasma CCK levels 30 min after their administration compared with 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 min, suggesting that the gastroprotective effect of CCK has physiological significance.

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

As shown in Fig. 3, the type A CCK receptor antagonist L-364718 at a dose of 1 mg/kg almost completely abolished the gastroprotective effects of orogastric oleate and SBTI. The lower dose of L-364718 (0.1 mg/kg) partially reversed oleate and SBTI-induced gastroprotection (66 ± 7 vs. 34 ± 8 and 77 ± 10 vs. 33 ± 9 mm²; P < 0.01). Type A CCK receptor blockade did not significantly increase gastric injury from acidified ethanol. L-364718 was also effective at preventing exogenous CCK-induced gastroprotection (94 ± 14 vs. 6 ± 3 mm²; P < 0.001), as previously reported (24). The

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**Table 2. Effects of SBTI on gastric mucosal blood flow before and after exposure to acidified ethanol**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GMBF, % baseline Before Irritant</th>
<th>GMBF, % baseline After Irritant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>87.9 ± 9</td>
<td>166.8 ± 17.9</td>
</tr>
<tr>
<td>SBTI, 100 mg</td>
<td>217.3 ± 30.9*</td>
<td>236.2 ± 48.8*</td>
</tr>
</tbody>
</table>

Gastric mucosal blood flow (GMBF) was recorded continuously with a laser-Doppler flow monitor in anesthetized model. After baseline was determined, transduodenal saline or SBTI was given and GMBF was recorded for 30 min. Acidified ethanol (150 mM HCl-50% ethanol) was administered intragastrically, and GMBF was recorded for 5 min. GMBF is reported as the average of the peak GMBF. Values are means ± SE; n = 5 for all groups. *P < 0.05 vs. saline counterpart. †P < 0.05 vs. saline before irritant.

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**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>
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<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 min without exposing the stomach to a luminal irritant. Orogastic saline, oleate, or SBTI were given for 30 min without exposure of the stomach to a luminal irritant. Plasma CCK levels are reported as means ± SE; n = 5/group; *P < 0.008 vs. saline counterpart.
Type B CCK receptor antagonist L-365260 did not have any inhibitory effects on the ability of CCK secretagogues to prevent acidified ethanol-induced gastric injury compared with the vehicle- or CCK secretagogue-treated animals (Fig. 4). However, L-365260 did prevent the gastroprotective actions of exogenous gastrin-17. 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-364718 on bile acid and 20% ethanol-induced gastric injury are shown in Fig. 5. In the absence of L-364718, 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-364718 also exacerbated gastric injury from these luminal irritants (6 ± 3 vs. 1.8 ± 0.4 and 7.5 ± 4 vs. 2.3 ± 0.4 mm²; P < 0.01), suggesting that endogenous CCK plays a role in maintaining mucosal integrity. L-364718 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-364718, exposure of the stomach to the mild irritant acidified taurocholate prevented gastric injury from acidified ethanol compared with control rats, consistent with adaptive cytoprotection. However, although L-364718 attenuated CCK- and CCK secretagogue-induced gastroprotection (Fig. 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.
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 Fig. 6. As shown, in the absence of L-NAME, both oleate and SBTI significantly increased GMBF and resulted in gastroprotection compared with saline-treated rats. Administration of the nonselective 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-min treatment with SBTI, a time point at which gastroprotection and hyperemia just became significantly increased (not shown). These results were not significantly different from those in Fig. 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). 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 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 Fig. 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 compared with 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 Figs. 8 and 9. As shown in Fig. 8, CCK and CCK secretagogues increased calcium-dependent NOS activity compared with rats receiving saline. In contrast, no change in calcium-independent NOS activity (NOS-1) 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). 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.

**Fig. 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 min without exposure of the stomach to a luminal irritant. NOS activity is reported as means ± SE; n = 5/group. *P < 0.05 for all groups vs. saline.

**Fig. 9.** Effects of saline, CCK, SBTI, and oleate on calcium-independent NOS activity in the crude homogenate from gastric mucosal membranous fractions was performed after 30-min treatment with saline, CCK, oleate, or SBTI. No significant differences were seen in any treatment groups compared with saline.

**DISCUSSION**

This study demonstrated that oleate and SBTI are potent gastroprotective agents against a variety of luminal irritants according to macroscopic and morphological criteria. These protective actions are mediated by activation of type A CCK receptors. Furthermore, both oleate and SBTI caused significant increases in plasma CCK levels that were similar in magnitude to those achieved with a gastroprotective dose of exogenous CCK. These findings suggest that another physiological 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 GMBF. Although Konturek et al. (16) and Heinemann et al. (11) demonstrated that exogenous CCK-induced hyperemia is reversed by nonselective NOS inhibition, 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 nonselective 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 nonspecific effects of L-NAME. In contrast to L-NAME, aminoxyanidine, a selective inhibitor of NOS-2, failed to reverse CCK-induced gastric hyperemia or CCK-induced gastroprotection. 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 flavin mononucleotide 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 Gly(2) 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, in regulating GMBF, 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 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 ~90% of NOS-3 resides within the membranous fraction of protein homogenates and only ~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, because 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 GMBF (30). Thus we believe our results indicate that exogenous CCK and CCK secretagogues increase activity of NOS-3, which causes a resultant increase in GMBF, preparing the gastric mucosa to withstand injury from a damaging luminal insult.

Our observations differ from those made by Brzozowski et al. (3). They found that CCK given intraperitoneally or intracerebrally resulted in increased NOS-2 mRNA in the gastric mucosa. 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 NOS-2. In contrast, we found no changes in NOS isoform immunoreactivity and that aminoxyanidine failed to reverse CCK-induced gastroprotection and hyperemia. Furthermore, aminoxyanidine 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 increased constitutive or calcium-dependent NOS activity in the membranous fraction of gastric mucosal homogenates compared with 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 30-min 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. (3). 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 analyses 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. (3). Although L-NAME abolished CCK-induced accumulation of nitrates and nitrites within the gastric lumen in their study, it is noteworthy that L-NAME preferen-
tially inhibits the constitutive NOS isoforms as op-
posed 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 amino-
guanidine, which has a 50-fold difference in affinity for
the mouse NOS-2 isoform vs. NOS-1 and a 500-fold
difference vs. NOS-3 (35), failed to reverse CCK-in-
duced gastroprotection or hyperemia further suggests
that NOS-2 does not play a role. Together, these find-
ings dispute the notion that NOS-2 mediates CCK-
induced gastroprotection and strongly suggest that
constitutive NOS isoforms are responsible for this ef-
f. In conclusion, this study demonstrated that CCK
secretagogues augment GMBF and are potent gastro-
protective agents against a variety of luminal irritants.
Type A CCK receptor blockade negated CCK secreta-
gogue-induced gastroprotection and exacerbated gas-
tric 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 gastro-
protective dose of exogenous CCK. These findings sug-
gest that CCK is released in response to a meal and
that it plays an important role in gastric mucosal
defense against injury from ingested ethanol or duode-
nal gastric reflux of bile. The mediator responsible for
CCK-induced gastroprotection appears to be nitric ox-
ide, because inhibition of NOS negated CCK secreta-
gogue-induced gastroprotection and hyperemia, effects
that were reversed by L-arginine but not D-arginine.
Moreover, exogenous CCK and CCK secretagogues in-
creased 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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