INTRODUCTION

Neurotensin (NT) is a tridecapeptide originally isolated from extracts of bovine hypothalami (Carraway and Leeman, 1973) and gastrointestinal tracts (Kitabgi et al., 1976; Carraway et al., 1978). The amino acid sequence is the same from both sources: pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH (Carraway and Leeman, 1975; Carraway et al., 1978). The presence of NT has also been demonstrated in the gastrointestinal tract of several other mammalian species including humans (Helmstaedter et al., 1977a; Sundler et al., 1977a; Draviam et al., 1990), rats (Carraway and Leeman, 1976a; Iwasaki et al., 1980), and dogs (Orci et al., 1976; Doyle et al., 1985). Neurotensin, as identified by HPLC, exhibited a 2-fold increase in plasma extracts following perfusion of the proximal ileum with a 10-mg sample of oleic acid, as compared with control samples of plasma collected before oleic acid perfusion. In whole-animal studies, the injection of a micellar solution of oleic acid into isolated segments of the duodenum resulted in elevated plasma immunoreactive NT in blood collected from the pancreaticoduodenal vein. Injection of a 1,000 mOsm sodium chloride solution had a slightly lesser and delayed effect compared with oleic acid, but a greater effect than 0.1 N hydrochloric acid in isotonic saline solution. Injection of an amino acid solution (10% Travasol), 300 mOsm glucose solution, or pure corn oil had no effect. These results demonstrate that intraduodenal oleic acid is a potent stimulus for the release of NT from the duodenum into the hepatic-portal circulation of chickens.

Key words: neurotensin, oleic acid, duodenum, chicken

ABSTRACT Peripheral and hepatic-portal plasma levels of neurotensin (NT) in fed and fasted chickens were determined using RIA. Portal levels of NT<sub>1–13</sub> (fed = 61.3 ± 3.9 fmol/mL; fasted = 44.5 ± 3.9 fmol/mL) were significantly higher than peripheral levels (fed = 8.2 ± 3.3 fmol/mL; fasted = 7.8 ± 3.0 fmol/mL) collected from the wing vein, indicating that some NT is metabolized in the liver. Portal plasma levels of NT collected from fed birds were also significantly higher than portal plasma levels of NT collected from fasted birds. Neurotensin, as identified by HPLC, exhibited a 2-fold increase in plasma extracts following perfusion of the proximal ileum with a 10-mg sample of oleic acid, as compared with control samples of plasma collected in the intestinal tract (Carraway and Leeman, 1976a) and the biologically active COOH-terminus of the peptide is highly conserved (Carraway et al., 1982).

Mammalian intestinal NT is localized in enteroendocrine cells, called N cells, which are found scattered in the mucosal layer of the jejunum. There are a few N cells in the ileum, but they are very scarce in the duodenum (Helmstaedter et al., 1977a; Sundler et al., 1977a). Near the base of the N cell in close proximity to capillaries are granules that release NT into the hepatic-portal circulation (Helmstaedter et al., 1977b; Sundler et al., 1977b) upon ingestion of a meal (Matchford et al., 1978; Hammer et al., 1982; Shaw and Buchanan, 1983). Relative to other digesta, lipids are the most potent in stimulating immunoreactive NT (iNT) release (Rosell and Rökäes, 1979; Ferris et al., 1981; Go and Denol, 1981; Flaten and Hanssen, 1982). Consumption of alcohol and coffee by humans (Matchford et al., 1978), or perfusion of rat intestine with amino acids, glucose, hypertonic saline, acidified saline, bile salt, and diluted bile, has had little or no effect on NT release (Ferris et al., 1981). In addition to intact NT (i.e., NT<sub>1–13</sub>), NT fragments such as NT<sub>1–8</sub> and NT<sub>1–11</sub> are also released or are formed as metabolites from NT<sub>1–13</sub> in response...
to lipids in the intestinal lumen (Ferris et al., 1985a; Draviam et al., 1990).

Using an RIA specific for bovine NT, Carraway and Bhatnagar (1980) have isolated and purified NT from chicken intestinal tracts and have demonstrated its amino acid sequence to be identical to bovine NT except for the substitution of His3, Val1, and Ala7. Most published research on intestinal NT has been based on mammals; very few studies have used birds (Komori et al., 1986; Denac and Scharrer, 1987; Rawson et al., 1990; DeGolier et al., 1997; Gui et al., 2000). However, there is more NT in the avian gut than in the mammalian gut (Carraway et al., 1982). The concentration of iNT in extracts of intestines measured using the most specific COOH-terminus directed antiserum, HC-8, gave measurements in chickens that were 10-fold higher than corresponding values for mammals. Also, in contrast to mammals, chicken N cells are distributed along the entire intestinal tract and are particularly numerous in the pylorus, the duodenal region that adjoins the gizzard. Avian N cells are also found in the colon, but are rare in the ceca and not present in the proventriculus or pancreas (Sundler et al., 1977b).

Based upon these unique aspects of avian NT, our objectives were to 1) determine the NT plasma levels from portal and peripheral sources in fed and fasted chickens and 2) inject isolated segments of the avian duodenum with nutrients to determine which components of a meal effectively release iNT into the circulation.

**MATERIALS AND METHODS**

**Birds**

Single Comb White Leghorn hens, 10 to 18 wk of age, were used in this study. Body weights ranged from 0.9 to 1.4 kg. The hens were housed in individual wire cages in environmentally controlled rooms maintained at 25°C. The RH was kept between 40 and 50%, and photoperiod was controlled to provide 14 h of light from 0900 to 2300 h. All birds had ad libitum access to water and a diet (all mash breeder ration: 16% protein, 4.5% crude fiber, 3.9% crude fat, 2,874 kcal/kg of ME, 2.7% calcium, and 0.8% phosphorus) prepared by the Animal Science Department of the University of Minnesota. All procedures were in accord with the policies of the University of Minnesota Committee on Animal Laboratory Care.

**Surgical Procedures**

Hens were fasted overnight to avoid meal-induced increases in plasma levels of NT. They were anesthetized with 40 mg/kg of sodium pentobarbital (Squibb-Marson, Cherry Hill, NJ) injected via the basilic vein. Abdominal feathers were plucked, and the skin was sterilized with alcohol. A 3-cm incision was made in the abdominal wall 1 cm below the left lateral notch of the sternum and 1 cm to the right of the linea alba. The skin and muscles layers were retracted to expose the duodenal loop, which was lifted out of the abdominal cavity for the duration of the experiment. Any apparent ingesta in the duodenum was gently massaged out of the duodenum into the ileum. The duodenal segment was then isolated by tying ligatures around the duodenum distal to the pylorus and proximal to the insertion of the pancreatic ducts. Care was taken to effectively close the lumen without transecting the gut.

The duodenal loop was then displaced laterally exposing its dorsal surface and the pancreaticoduodenal (PD) vein. The PD vein was cannulated with an intravenous catheter (24 gauge × 1.9 cm; Critikon, Tampa, FL), sutured in place, and a sample of blood (3 mL) was drawn as a control (pretreatment) sample. Heparinized (1%) saline was injected into the venous catheter to prevent clotting. The chickens remained anesthetized throughout the entire experiment, and body temperature was kept constant with a heating pad.

**Treatment**

The duodenal segment was then injected with 2.5 mL of one of the following test solutions, which represent a typical digestive or secretory constituent in the duodenum: (1) 0.1 N hydrochloric acid in isotonic saline solution, (2) an amino acid solution (10% Travasol, Baxter Healthcare Corporation, Deerfield, IL), (3) 1,000 mOsM sodium chloride solution (to mimic the osmolarity of the 0.1 N HCl solution), (4) 300 mOsM glucose solution, (5) pure corn oil (Mazola, Englewood Cliffs, NJ), and (6) a micellar solution of oleic acid composed 0.45 g of taurodeoxycholate sodium salt (Sigma-Aldrich, St, Louis, MO) in 100 mL of physiological saline with 0.1 mL of oleic acid (Sigma-Aldrich, St. Louis, MO). Oleic acid (C18H34O2) was used as the lipid constituent because it is probably the most common fatty acid occurring in natural fats (Mayes, 1988). All solutions introduced into the duodenum were previously warmed to the body temperature of chickens (41°C) in a water bath.

Posttreatment blood samples (3 mL) were collected at 30 and 60 min after injection of a nutrient into the duodenum. All blood samples were collected in prechilled tubes (Corning Glass Works, Corning, NY) containing 50 μL of EDTA (Sigma-Aldrich, St. Louis, MO) at a concentration of 60 mg/mL and adjusted to pH 7, and 3 μL of 0-phenanthroline (Sigma-Aldrich) solubilized in dimethylsulfoxide to a concentration of 0.3 M. Samples were then centrifuged, and the plasma was removed and frozen at −70°C until assayed.

In a separate group of chickens, portal and peripheral plasma samples from the wing vein or the PD vein were collected and treated as described previously. To obtain preprandial or fasting plasma levels of NT, chickens were starved for 16 h before sampling. For postprandial or fed plasma levels of NT, chickens were allowed to eat for ad libitum 2 h before sample collection.
**NT Assays**

Radioimmunoassays for chicken NT (Carraway and Leeman, 1976b) using $^{125}$I-labeled NT and multiple region-specific rabbit antisera primarily directed toward the COOH-terminal region (Carraway and Leeman, 1975) were employed. Several substances in plasma, however, cross-react with region-specific antisera. Thus, samples were first isolated using HPLC and then subjected to RIA for several region-specific antisera (Carraway et al., 1980).

**iNT in Intestinal Extracts and Plasma**

Immunoreactive NT is present along the entire length of the small intestine of chickens (Carraway and Bhatnagar, 1980) and represents a storehouse of NT in the tissue. Some of that NT is normally released into the circulation and is present as iNT in the plasma (Ferris et al., 1981). Because avian NT was assayed from hepatic-portal plasma, it was first necessary to determine how much iNT was present in the plasma before the introduction of oleic acid into the isolated intestinal segments. Thus, a 10-mg sample of intestinal mucosa taken from the proximal 10 cm of the chicken ileum and 6 mL of hepatic-portal plasma were taken before, and 30 min after, oleic acid perfusion. These samples were extracted with acid/acetone and applied to reverse-phase HPLC (Carraway and Leeman, 1976b; Carraway et al., 1980).

**Statistical Analysis**

Repeated measures ANOVA (SAS Institute, 1992) were used to determine if any significant differences existed between the levels of iNT released into the hepatic-portal circulation and the time following the injections of the different test solutions into the duodenum. Analysis of variance was also used to determine if portal and peripheral plasma levels of NT collected from fed and fasted birds were significantly different from each other. These differences were considered significant at $P < 0.05$.

**RESULTS**

**Plasma Levels of NT**

Hepatic portal plasma levels of NT were significantly higher than peripheral plasma levels of NT (Table 1). Thus, the majority of NT released from the gut is broken down in its first pass through the liver. Hepatic-portal plasma levels of NT collected from fed sources were also significantly higher than portal plasma collected from fasted birds ($P < 0.05$), indicating that the direct presence of food in the gut stimulates the release of NT into the hepatic-portal circulation. The peripheral plasma levels of NT collected from fed and fasted birds were not significantly different.

<table>
<thead>
<tr>
<th>Source</th>
<th>Mean ± SE</th>
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<tr>
<td>Peripheral</td>
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<tr>
<td>Fed</td>
<td>8.2 ± 3.3a</td>
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<td>Fasted</td>
<td>7.8 ± 3.0b</td>
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<td>Portal</td>
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<tr>
<td>Fed</td>
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<tr>
<td>Fasted</td>
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Means with different superscripts are different ($P < 0.05$).

**Immunoreactive NT Profiles**

Comparison of iNT profiles obtained during reverse-phase HPLC (Figure 1) indicates that after 30 min of retention time, a peak of 2.5 pmol/mL eluted from the ileum extract (Figure 1A), whereas only a peak of 0.04 pmol/mL eluted from the control plasma in the same region after 30 min (Figure 1B). Thus, the small intestine contributed very little NT into the portal circulation under basal conditions. The peak from NT, however, rose from 0.04 to 0.075 pmol/mL in the plasma extracts that were collected following perfusion of the ileum with oleic acid (Figure 1C). This is almost a 2-fold increase in iNT concentration, which demonstrates that intraduodenal lipid is a potent stimulus for the release of NT from the duodenum into hepatic-portal circulation.

**Release of NT in Response to Intraluminal Contents**

Plasma concentrations of iNT following the injection of the test solutions into the duodenum were calculated as a percentage of the pretreatment plasma level in each bird. Even though chickens were fasted overnight before experiments, the pretreatment plasma levels were somewhat variable but were not significantly different from each other. It can be speculated that ingesta retained in the crop or refluxed from the lower intestine might account for ingesta occurring in the duodenum of fasted hens and contribute to the release of iNT into the portal plasma before treatments.

There were significant interactions between the levels of iNT released by exposure of the duodenal mucosa to the various treatment solutions and the amount of time that the solutions were kept in the duodenal segment (Figure 2). Thirty minutes after injection of oleic acid, iNT levels were significantly greater ($P < 0.05$) than those resulting from injections of amino acids, glucose, saline, and corn oil, but not HCl. Sixty minutes after injection of oleic acid, iNT levels were still higher than saline and were statistically higher than amino acids, HCl, glucose, and corn oil. Although oleic acid was the most potent stimulus used, the iNT levels 60 min after injection of hypertonic saline were also statistically higher ($P < 0.05$) than amino acids, glucose, and corn oil. Immunoreactive NT was also elevated 60 min after HCl injection and was greater than corn oil ($P < 0.05$).
The results of this study have demonstrated that increased levels of iNT are released into the hepatic-portal circulation in response to the presence of a micellar solution of oleic acid in contact with the duodenal mucosa. Hypertonic saline and HCl had somewhat reduced and delayed effects compared with oleic acid, but their effects were greater than the effect of amino acids, glucose, and corn oil. The saline test solution was hypertonic to the glucose solution, suggesting that osmolarity may be a variable stimulating the release of NT. However, the amino acid solution was isosmotic to the saline and yet produced an even smaller response, indicating that osmolarity is not a factor in NT release.

Our results also indicated that other factors besides nutrient stimulation may regulate the release of NT in chickens. The profile of iNT obtained from HPLC (Figure 1) showed that iNT is present in the plasma before any intraduodenal nutrient stimulation. Results from the RIA in Table 1 indicate NT exists in portal plasma collected from fasted birds, but at levels that are significantly lower than NT plasma levels from fed birds. Because all of our birds responded to some degree to the presence of a nutrient along the gut mucosa, our results are consistent and in agreement with mammalian studies, which demonstrated that oleic acid is the most potent nutrient in stimulation of the release of NT into portal circulation (Rosell and Rökaeus, 1979; Ferris et al., 1981; Go and Demol, 1981; Flaten and Hanssen, 1982; Walker et al., 1985).

In addition to NT, NT fragments or metabolites of NT can also be released into the circulation after lipid infusion (Ferris et al., 1981). Estimating the amount of NT released in response to a physiologic stimulus such as intraintestinal oleic acid is complicated by the slow elimination of NT metabolites from circulation. Aronin et al. (1982) has shown that a portion of NT$^{1-13}$ that is injected intravenously into a rat is rapidly metabolized to NT$^{1-8}$ or NT$^{1-11}$. These NT metabolites have a longer half-life (5 and 9 min, respectively) than the whole peptide (i.e., NT$^{1-13}$ $t_{1/2} = 0.55$ min). The amount of NT$^{1-13}$, its amino terminal fragments (NT$^{1-8}$ and NT$^{1-11}$), and its carboxyl terminal fragments (NT$^{8-13}$) can be identified by HPLC and measured by RIA (Ferris et al., 1985b; Draviam et al., 1990). Because we were measuring NT$^{1-13}$ and wanted to avoid significant contamination with NT$^{1-8}$ and NT$^{1-11}$, we collected

**DISCUSSION**

The results of this study have demonstrated that increased levels of iNT are released into the hepatic-portal circulation in response to the presence of a micellar solution of oleic acid in contact with the duodenal mucosa. Hypertonic saline and HCl had somewhat reduced and delayed effects compared with oleic acid, but their effects were greater than the effect of amino acids, glucose, and corn oil. The saline test solution was hypertonic to the glucose solution, suggesting that osmolarity may be a variable stimulating the release of NT. However, the amino acid solution was isosmotic to the saline and yet produced an even smaller response, indicating that osmolarity is not a factor in NT release.

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**Figure 1.** Comparison of the profiles of immunoreactive neurotensin (NT) obtained during reverse-phase HPLC of (A) an extract of 10 mg of chicken ileum; (B) an extract of 6 mL of control plasma; and (C) an extract of 6 mL of plasma obtained 30 min after lipid (here, oleic acid) perfusion into the ileum. The dotted line in panel A represents the acetonitrile line.

**Figure 2.** Immunoreactive neurotensin (iNT; fmol/mL) in hepatic-portal plasma following injections of amino acids (aa, n = 8), HCl (n = 7), glucose (gluc, n = 7), hypertonic NaCl (saline, n = 6), oleic acid (oleic, n = 7), and corn oil (corn, n = 6) into isolated segments of the duodenum. Data points (means ± SEM) are presented as a percentage of the pretreatment plasma level (hepatic-portal, fasted) in each bird. Several interactions occurred between the levels of iNT released into the hepatic-portal circulation and the time following the injections of the different test solutions into the duodenum. See the Results section for individual relationships.
plasma samples directly from hepatic-portal blood via the pancreaticoduodenal vein before NT was metabolized in the liver. Ferris et al. (1985b) demonstrated in rats that there is significantly more NT in the portal circulation than in the peripheral circulation during lipid perfusion in the small intestine.

Rosell and Rökaeus (1981), after administering a lipid solution to humans (intravenously), could not detect any significant change in circulating NT. This prompted several studies to determine if NT release is dependent upon direct stimulation of the mucosa of the intestinal segments containing the N cells. Despite the fact that the major stores of mammalian NT are in the ileum (Helmstaedter et al., 1977a; Sundler et al., 1977a), the selective perfusion of the duodenum and jejunum, and not the ileum, significantly increased the portal plasma levels of NT (Fujimura et al., 1989; Kihl et al., 1980; Read et al., 1984; Walker et al., 1985). Thus, in mammals, the release of NT from the distal gut may not be due to direct stimulation, but may be dependent upon a signal from the proximal gut and mediated via a local nerve or peptide intermediate (Walker et al., 1985; Fujimura et al., 1989). In chickens, it appears that direct stimulation is responsible for some NT release, but our conclusions are based only on the response from the duodenal mucosa. Because N cells are located along the entire intestine of the chicken (Helmstaedter et al., 1977a; Sundler et al., 1977a), further study is needed to determine if the ileum is equally effective in releasing NT and if the response is dependent upon some sort of signaling or feedback.

Some mammalian studies indicate that the release of NT into the circulation is lipid specific. For example, in primary cultures of canine enteric endocrine cells, the release of NT-like immuinity (NTLI) was demonstrated to be specific for long-chain, unsaturated fatty acids, and stereospecific for cis isomers (Barber et al., 1991). Perfusion of rat intestine with fatty acids with 4 or more carbons and alcohols with 2 or more carbons significantly elevated NTLI plasma levels (Ferris et al., 1985a). In the present study, corn oil, which is composed of a mixture of several glycerides of fatty acids and unsaturated fatty acids, was the least stimulatory in releasing iNT (Figure 2). Even though oleic acid is present in corn oil, the percent is variable (19 to 49%, O’Neil, 2006) and it was not administered with bile salts or as a micellar solution. Mixing the corn oil with bile, exposing it to a different segment of the gut, or using a bird species acclimated to a higher fat content in their diet may yield different results.

In contrast to some mammalian studies (Ferris et al., 1981; Fujimura et al., 1989), our results indicate that hypertonic saline is an effective stimulus in releasing iNT into the hepatic-portal plasma. The test solutions used in this experiment are similar to the chemical nature of digesta in the duodenum and can depress gastric motility; this is known as the enterogastric reflex. When hydrochloric acid, hypertonic saline, and lipid solutions were injected into the turkey duodenum, gastric motility was inhibited (Duke and Evanson, 1972; Duke et al., 1973). The onset of inhibition after the acid injection was within seconds, but the onset of inhibition following lipid or saline injection occurred 4 to 6 min later. Because lipids and hypertonic saline release NT, the enterogastric inhibitory effects of lipids and hypertonic saline in chickens may be mediated humorally by NT. Evidence in support of this mechanism is that NT has been shown to inhibit gastric motility in chickens (DeGolier et al., 1997).

In conclusion, our data suggest that the N cells in the chicken intestine appear to function as a production, intracellular storage, and release site for NT into the portal circulation. The elevation of plasma iNT following direct stimulation by a micellar solution of oleic acid demonstrates that NT has a physiological role in avian digestion as it does in mammals. The localization of NT in the gut as well as in the brain make it a member of a rapidly growing list of brain-gut peptides (e.g., gastrin, cholecystokinin, motilin, vasoactiveintestinal peptide, substance P, somatostatin, and gastrin-releasing peptide). When localized in brain or gut neurons, these peptides are known to be synaptic mediators or neurotransmitters; when localized in the gut, they act as gastrointestinal hormones and employ their effects in an endocrine or paracrine fashion.

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