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Note

Characterization of fat-induced neurotensin-like immunoreactivity in plasma using column liquid chromatography and radioimmunoassay

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Neurotensin is a tridecapeptide isolated and characterized by Carraway and Leeman in 1973 [1]. Although small amounts of neurotensin have been detected in the esophagus, stomach, duodenum and colon, the primary sites of neurotensin localization are the jejunum and ileum [2,3]. Neurotensin cells appear to be true endocrine cells and they have been identified in the small intestine of man and animals by histochemical techniques [4]. Neurotensin can stimulate pancreatic exocrine secretion [5,6], and it also inhibits gastric acid secretion in dogs and in man [7,8]. Ingestion of fat is the most potent stimulus for the release of neurotensin in dogs, rats and man [9–11].

The objectives of this study were to develop a high-performance liquid chromatographic (HPLC) procedure for separating neurotensin$^{1-13}$ and neurotensin fragments (i.e. neurotensin$^{1-8}$, neurotensin$^{1-11}$, neurotensin$^{8-13}$ and to measure these fragments in plasma obtained from dogs given intraduodenal fat.

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EXPERIMENTAL

Chemicals

The following chemicals and supplies were used in this study: HPLC-grade acetonitrile and ultrapure water (Burdick and Jackson Labs., Muskegon, MI, U.S.A.), trifluoroacetic acid (TFA) (Pierce, Rockford, IL, U.S.A.), ammonium acetate (Fisher Scientific, Fair Lawn, NJ, U.S.A.), Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.), Millex-HV_4 filter units (Millipore, Bedford, MA, U.S.A.), radiolabeled $Na^{125}I$ (Amersham, Arlington Heights, IL, U.S.A.), chloramine-T (Eastman, Rochester, NY, U.S.A.), sodium metabisulfite (Mallinckrodt, New York, NY, U.S.A.), heparin (Liquamin, Organon Diagnostics, West Orange, NJ, U.S.A.), Lipomul (Upjohn Pharmaceuticals, Kalamazoo, MI, U.S.A.), aprotinin (Novo Research Institute, Bagsvaerd, Denmark).

Neurotensin$^{1-13}$ and neurotensin fragments $^{1-8}$, $^{1-11}$, $^{8-13}$ were obtained from Bachem (Torrance, CA, U.S.A.).

Apparatus

A Model 342 gradient liquid chromatograph (Beckman Instruments, Berkeley, CA, U.S.A.) equipped with a Model 420 system controller, to 112 solvent delivery modules and a Model 160 variable-wavelength UV detector were used in this study. A Wisp 710B automatic sampler (Waters Assoc.) was used for the injection of plasma extracts and standards. A Nova-Pak C_{18} (4 μm particle size, 15 cm$\times$3.9 mm) analytical column (Waters Assoc.) was used for the reversed-phase separation of neurotensin and neurotensin fragments.

Experimental design

Six mongrel dogs were fasted for 18 h with access to water before the following experiment. Lipomul (corn oil, a triglyceride) (2 g/kg per h in 50% saline) was infused through a duodenal cannula at a rate of 100 ml/h for 1 h using a Harvard pump (Harvard Apparatus, South Natick, MA, U.S.A.). Peripheral blood (20–22 ml) was collected at 0 (basal), 15, 30 and 60 min into glass tubes containing heparin (15 U) and aprotinin (100 kallikrein inhibitory units per ml of blood). Plasma was immediately separated by centrifugation (1500 *g*) and stored at $-20°C$ until extracted for neurotensin-like immunoreactivity (NTLI).

Extraction of neurotensin from plasma and HPLC separation

Plasma neurotensin and neurotensin fragments were extracted by means of a Waters Sep-Pak rack (Waters Assoc.). C_{18} Sep-Pak cartridges were prewashed sequentially with 10 ml of acetonitrile and 10 ml of 0.05 *M* ammonium acetate (pH 5.5). Plasma was then added (4 ml) and washed with 10 ml of ammonium acetate. NTLI was eluted with 3 ml of acetonitrile–ammonium acetate (1:1). Extracts representing 12 ml of plasma (same original specimen) were combined and evaporated to dryness under nitrogen at 30°C. The residues were reconstituted in 400 μl of HPLC-grade water and filtered through a 0.45-μm Millex-HV_4 filter: 200 μl of the filtrate was then injected into the chromatograph.

HPLC analysis was carried out at a flow-rate of 1 ml/min, and UV absorption

was monitored at 214 nm. A stepped gradient consisting of 0.05% TFA in water (solvent A) and acetonitrile–0.05% aq. TFA (9:1) (solvent B) was used for the reversed-phase separation (9–13% solvent B from 0 to 6 min, 13–26% solvent B from 6 to 12.5 min and 26–41% solvent B from 12.5 to 47.5 min). The retention times (t_R) for neurotensin and neurotensin fragments were characterized by injecting pure standards. The HPLC effluents were collected in 1-ml fractions using an LKB 2111 multirac fraction collector (LKB-Producter, Bromma, Sweden). Fractions were dried under nitrogen gas, reconstituted in neurotensin assay buffer (0.01 *M* potassium phosphate, 0.15 *M* sodium chloride, 0.1% sodium azide and 2% normal rabbit serum, pH 7.5) and analyzed by radioimmunoassay (RIA) for neurotensin.

Neurotensin radioimmunoassays

The RIA and iodination procedures have been described in detail previously [6]. We utilized three different neurotensin antisera in this study. Neurotensin antiserum 193 has been characterized previously [6]; it was used to quantitate neurotensin levels in the HPLC fractions containing neurotensin$^{1-13}$ (t_R=19.8 min). Antiserum 205 against neurotensin$^{1-13}$ (generated in our laboratory) was used to measure the HPLC fractions containing the C-terminal fragment, neurotensin$^{8-13}$ (t_R=16.4). Another neurotensin antiserum (generous gift of J.S. Kizer, University of North Carolina, Chapel Hill, NC, U.S.A.), which is directed towards the midregion of neurotensin (amino acids 5–9) [12], was used to measure the HPLC fractions containing the N-terminal neurotensin fragments, neurotensin$^{1-8}$ (t_R=11.1) and neurotensin$^{1-11}$ (t_R=14.3).

Statistics

Results are expressed as the mean ± S.D. The Friedman test [13] was used to analyze data for statistical differences between means. Differences with a $p < 0.05$ were considered significant.

RESULTS

Fig. 1 shows the HPLC separation of neurotensin$^{1-13}$ and neurotensin fragment standards. The retention times for neurotensin$^{1-8}$, neurotensin$^{1-11}$, neurotensin$^{8-13}$ and neurotensin$^{1-13}$ are characterized as 11.1, 14.3, 16.4 and 19.8 min, respectively.

In order to determine the efficiency of the Sep-Pak extraction procedure, fasted dog plasma was spiked with graded amounts (6.2, 12.5, 25, 50, 100 and 200 pg/ml) of neurotensin$^{1-13}$ and was extracted using the neurotensin extraction procedure. The extracts were then assayed by the neurotensin RIA. The recovery of added neurotensin$^{1-13}$ using the Sep-Pak extraction procedure is shown in Fig. 2. The recovery of neurotensin was 90%. The recovery of neurotensin$^{1-13}$ (37.5 pg/ml, added to dog plasma) after the Sep-Pak extraction procedure through the analytical HPLC column was 72%.

The mean plasma levels of neurotensin$^{1-8}$, neurotensin$^{1-11}$, neurotensin$^{8-13}$ and neurotensin$^{1-13}$ in response to intraduodenal (i.d.) fat, as determined by our

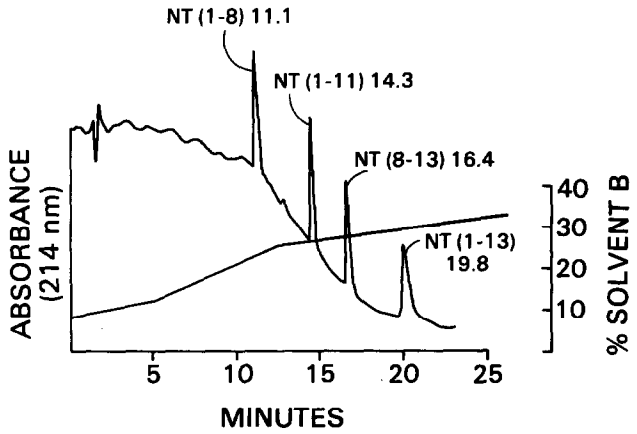

Fig. 1. HPLC separation of a standard mixture of neurotensin (NT) and NT fragments: NT $^{1-8}$ (500 ng), NT $^{1-11}$ (250 ng), NT $^{8-13}$ (500 ng) and NT $^{1-13}$ (500 ng). Retention times in minutes are shown after each form.

HPLC–RIA procedure, are shown in Fig. 3. A significant increase ($p<0.05$) in the circulating levels of neurotensin $^{1-13}$, neurotensin $^{1-8}$ and neurotensin $^{1-11}$ was observed at 15, 30 and 60 min when compared to basal levels. Although neurotensin $^{8-13}$ levels increased during i.d. infusion of fat, they failed to achieve statistical significance ($p<0.05$). Fig. 4 shows neurotensin levels in plasma that has not been subjected to HPLC separation. Plasma neurotensin levels were quantitated with an antiserum that fails to detect neurotensin $^{1-8}$, neurotensin $^{1-11}$ or neurotensin $^{8-13}$, but does detect neurotensin $^{1-13}$. As expected, neurotensin levels are comparable to those levels observed in plasma extracts subjected to HPLC separation.

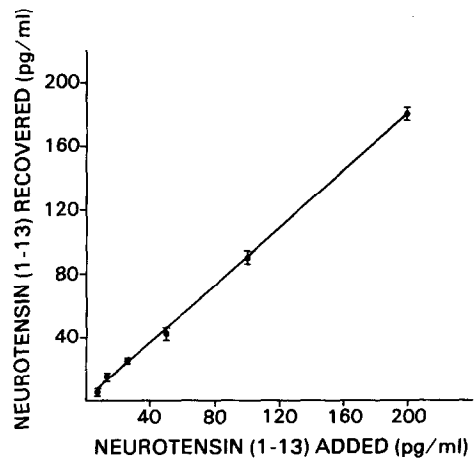

Fig. 2. Relationship between amounts of NT $^{1-13}$ added to and recovered from dog plasma. Known amounts of NT $^{1-13}$ were added to dog plasma and then separated from plasma by the Sep-Pak extraction procedure. The recovered NT $^{1-13}$ was quantitated by the neurotensin RIA.

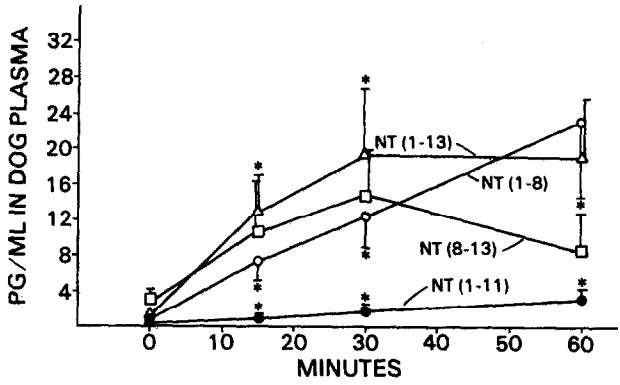

Fig. 3. NT $^{1-13}$, NT $^{1-8}$, NT $^{1-11}$ and NT $^{8-13}$ levels in canine plasma (six dogs) during infusion of i.d. lipomul as determined by HPLC-RIA ($* = p < 0.05$ versus 0 min).

DISCUSSION

The present study shows that neurotensin $^{1-13}$ and N-terminal fragments of neurotensin increase significantly during i.d. infusion of fat in dogs. Neurotensin $^{8-13}$ levels failed to rise significantly. Authentic neurotensin $^{1-13}$ appears to be present in plasma collected from dogs given i.d. fat; this finding confirms our preliminary report [14]. The progressive increase of concentrations of neurotensin $^{1-8}$ and neurotensin $^{1-11}$ during the infusion suggests that they are breakdown products of neurotensin $^{1-13}$. N-Terminal fragments of neurotensin (i.e. neurotensin $^{1-8}$ and neurotensin $^{1-11}$) are biologically inactive [15].

Earlier studies show that exogenously infused neurotensin $^{1-13}$ is metabolized rapidly to its N- and C-terminal fragments in rats [16]. In vitro experiments have shown that incubation of neurotensin $^{1-13}$, $^{1-8}$ and $^{8-13}$ in plasma resulted in stable levels of N-terminal fragments and a complete disappearance of C-termi-

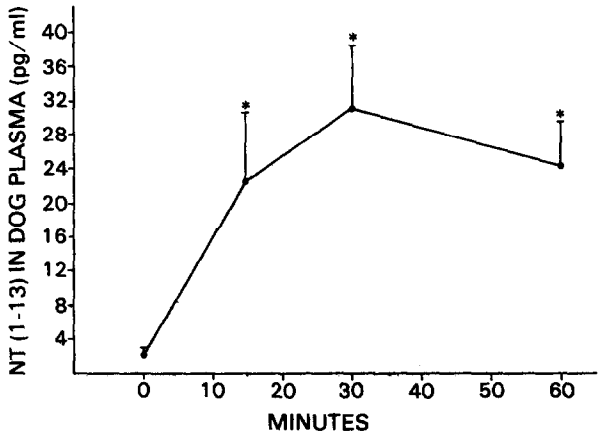

Fig. 4. NT $^{1-13}$ levels in plasma extracts not subjected to HPLC separation as determined by radioimmunoassay using antibody 193 ($* = p < 0.05$ versus 0 min).

nal fragments [17], which indicates that C-terminal fragments are destroyed much faster than N-terminal fragments.

Shulkes et al. [18] have shown that the major organs that metabolize neurotensin are the kidney, brain and gut, and that there is a greater clearance of C-terminal than of N-terminal fragments.

Intact neurotensin$^{1-13}$ has been previously found to increase in human plasma in response to oral feeding [19]. Neurotensin$^{1-13}$, however, was found to be a minor component of neurotensin immunoreactivity in human plasma, the major components being the N-terminal fragments, neurotensin$^{1-8}$ and neurotensin$^{1-11}$. In the present study, we have fractionated the components into neurotensin$^{1-13}$ and its metabolic components, neurotensin$^{1-8}$, neurotensin$^{1-11}$ and neurotensin$^{8-13}$. In contrast to the earlier finding in humans [19], neurotensin$^{1-8}$ and neurotensin$^{1-13}$ were found in roughly equal amounts in the plasma of dogs, with a smaller concentration of neurotensin$^{1-11}$. We have also found high levels of neurotensin$^{8-13}$ in the peripheral dog plasma.

Neurotensin levels in human plasma have been measured using RIA without HPLC separation [20–22]. In these studies, neurotensin levels were significantly higher than the levels we have reported, since direct determination of plasma or of a plasma extract yields the total radioimmunoassayable neurotensin. Values in these human experiments reflect some magnitude of interference of unidentifiable plasma factors present in plasma.

ACKNOWLEDGEMENTS

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