Original Article

Effect of Enterostatin on Insulin, Glucagon, and Somatostatin Secretion in the Perfused Rat Pancreas

Ramona A. Silvestre, Jovita Rodríguez-Gallardo, and José Marco

Enterostatin is a pentapeptide generated by trypic digestion of procolipase in the small intestine. Both peripheral and central administration of this peptide to rats has been shown to reduce food intake, this reduction being due to specific suppression of fat intake. In perifused pancreatic rat islets, enterostatin has been shown to inhibit the insulin response to a high glucose concentration. In the present study, we have investigated the effect of exogenous enterostatin on insulin, glucagon, and somatostatin secretion by the isolated perfused rat pancreas. Enterostatin, at 100 nmol/l, inhibited the insulin response to 9 mmol/l glucose (by 70%), 0.1 mmol/l tolbutamide (by 40%), and 5 mmol/l arginine (by 70%). Enterostatin had no effect on glucagon and somatostatin release at a maintained glucose level (5.5 mmol/l) or in response to 5 mmol/l arginine. Finally, preinfusion of the rat pancreas with a high enterostatin concentration (500 nmol/l) did not alter the insulin response to glucose, an observation that would rule out a toxic effect of this peptide on the β-cell. In summary, in the perfused rat pancreas, enterostatin, at putatively physiological concentrations, inhibits insulin secretion without affecting glucagon or somatostatin output, thus pointing to a direct effect of enterostatin on the β-cell and not through an α-cell or δ-cell paracrine effect. Because enterostatin is generated in the small intestine after feeding, it might play a role in the enteroinsular axis as an anti-incretin agent. *Diabetes* 45:1157–1160, 1996

Colipase, a cofactor of pancreatic lipase, is generated in the small intestine by tryptic digestion of its precursor, procolipase, which is synthesized by the exocrine pancreas and secreted in the pancreatic juice (1). As a result of tryptic digestion of procolipase in the small intestine, besides colipase, a pentapeptide is generated which sequence corresponds to Val-Pro-Asp-Pro-Arg (2,3). Both peripheral and central administration of this pentapeptide to rats has been shown to reduce food intake (4,5), this reduction being due to specific suppression of fat intake (6). Erlanson-Albertsson et al. (7) observed that in pigs, intraduodenal infusion of this novel pentapeptide inhibited exocrine pancreatic protein secretion, and they proposed the term enterostatin to designate it (7).

Interestingly, some peptides with anorectic effect, such as amylin and calcitonin (8–11), also behave as inhibitors of insulin secretion (12,13). In this context, it has recently been shown that enterostatin inhibits insulin release induced by a high glucose concentration in perifused pancreatic rat islets (14). To further explore the influence of enterostatin on islet cell function, we have investigated its effect on insulin, glucagon, and somatostatin secretion in the isolated perfused rat pancreas.

RESEARCH DESIGN AND METHODS

Fed male Wistar rats (200–225 g body wt) were used as donors. After the rat was anesthetized with pentobarbital sodium (50 mg/kg, i.p.), the pancreas was dissected and perfused in situ according to the procedure of Leclercq-Meyer et al. (15), as adapted in our laboratory (16). Effluent samples were collected from the portal vein, without recycling, at 1-min intervals (flow rate, 2 ml/min) and frozen at -20°C until the time of assay. The perfusion medium consisted of a Krebs-Henseleit buffer: 115 mmol/l NaCl, 4.7 mmol/l KCl, 2.6 mmol/l $CaCl_2$, 1.19 mmol/l H_2KPO_4, 1.19 mmol/l $MgSO_4 \cdot 7H_2O$ and 24.9 mmol/l $HNaCO_3$ (gas phase 95:5, O_2:CO_2; pH 7.4), supplemented with 4% (wt/vol) dextran T-70 (Pharmacia LKB Biotechnology, Uppsala, Sweden), 0.5% (wt/vol) Cohn Fraction V bovine albumin (Sigma, St. Louis, MO), and 5.5 mmol/l glucose (Sigma). Synthetic rat enterostatin (Val-Pro-Asp-Pro-Arg, 73% purity), purchased from Peninsula Laboratories (Belmont, CA), was dissolved in 0.9% NaCl containing 0.1% bovine albumin (Cohn Fraction V). This solution (1.78 μmol/l) was prepared daily, immediately before experiments. When added to the perfusate, it gave a final concentration of 100 nmol/l enterostatin. After a 35-min equilibration period, baseline samples were collected for 5 min. Rat enterostatin, with or without addition of other β-cell secretagogues—9 mmol/l glucose, 5 mmol/l L-arginine hydrochloride (Sigma), and 0.1 mmol/l tolbutamide (sodium tolbutamide, Upjohn, Kalamazoo, MO)—was infused through a sidearm cannula, as described in the corresponding figures.

In another series of experiments, we examined the response of the β-cells subjected to enterostatin preinfusion to glucose. For this purpose, 500 nmol/l enterostatin plus 9 mmol/l glucose were infused for 10 min and, after a wash-out period of 10 min with the initial perfusate, 9 mmol/l glucose alone was used for 10 min as β-cell secretagogue. In control experiments, 9 mmol/l glucose was infused for two 10-min periods, following a similar protocol. Insulin (17,18), glucagon (19), and somatostatin (20) were measured by radioimmunoassay. Anti-pig insulin serum (I8510, Sigma) and rat insulin standards (Novo Nordisk, Denmark) were used. Anti-glucagon serum (Unger's 30K) and antisomatostatin serum (Unger's 80C) were donated by R.H. Unger (University of Texas, Health Sciences Center, Dallas, TX). All samples for each series of experiments were analyzed within the same assay. Results are presented as the mean ± SE. Hormone response was calculated as the integrated area of the curve above the mean preinfusion level (average of all the baseline levels for each perfusion) using the trapezoidal method. The normal distribution of our data was demonstrated by the Kolmogorov-Smirnov test (21). Differences between values were tested

From the Puerta de Hierro Hospital and Department of Physiology, Autónoma University of Madrid, Madrid, Spain.

Address correspondence and reprint requests to Dr. J. Marco, Hospital Puerta de Hierro, Universidad Autónoma de Madrid, San Martín de Porres, 4, 28035 Madrid, Spain.

Received for publication 11 January 1996 and accepted in revised form 28 April 1996.

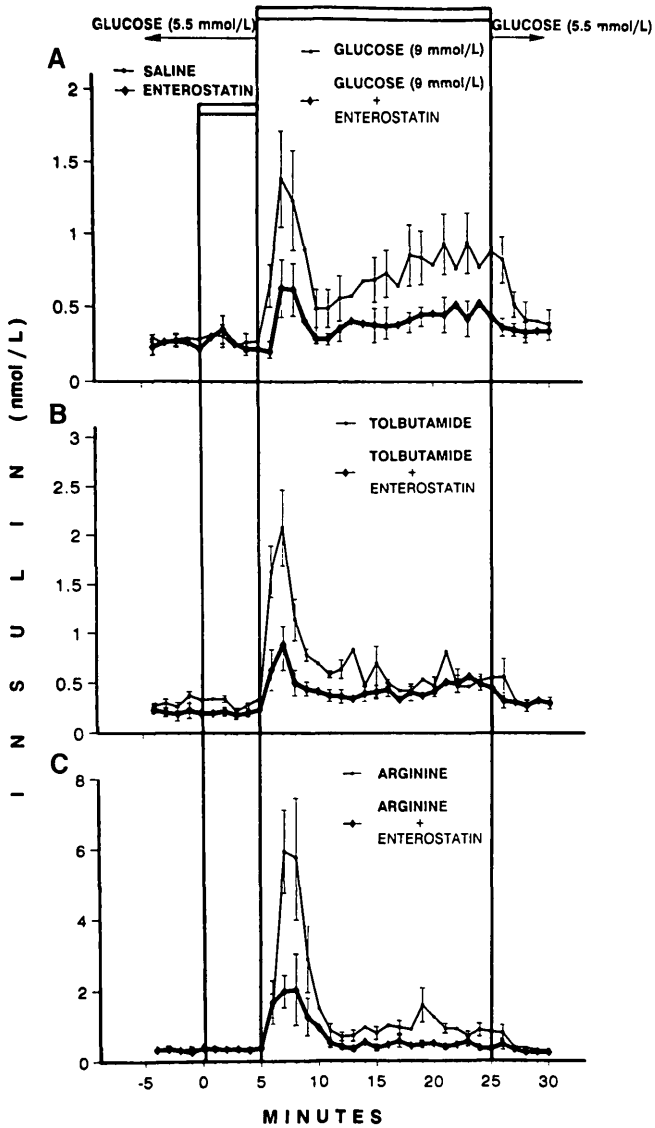

FIG. 1. Effect of 100 nmol/l rat enterostatin on insulin secretion in the perfused rat pancreas. Squares correspond to control experiments: from 0 to 5 min, saline infusion; from 5 to 25 min, 9 mmol/l glucose infusion (n = 5, A), 0.1 mmol/l tolbutamide infusion (n = 5, B), or 5 mmol/l arginine (n = 6, C). Rhombi correspond to enterostatin experiments: from 0 to 5 min, 100 nmol/l enterostatin infusion; from 5 to 25 min, glucose plus enterostatin infusion (n = 5, A), tolbutamide plus enterostatin infusion (n = 5, B), and arginine plus enterostatin infusion (n = 6, C). Data are means ± SE.

for significance by analysis of variance and by the Student's t test for unpaired samples.

RESULTS

The effect of 100 nmol/l rat enterostatin on insulin secretion at 5.5 and 9 mmol/l glucose is shown in Fig. 1*A*. Enterostatin did not significantly modify insulin secretion at 5.5 mmol/l glucose ($F_{5,40}$ = 1.23) but clearly inhibited the insulin response induced by an increase in the glucose concentration of the perfusate from 5.5 to 9 mmol/l (incremental area: 6.6 ± 3 pmol/20 min vs. 20 ± 5 pmol/20 min in control experiments, $P < 0.05$). Both phases of insulin secretion were suppressed during enterostatin infusion.

As shown in Fig. 1*B*, the insulin release evoked by 0.1 mmol/l sodium tolbutamide was reduced by 100 mmol/l enterostatin (incremental area: 9.8 ± 1.5 pmol/20 min vs. 17 ± 2.7 pmol/20 min in control experiments, $P < 0.05$), this reduction mainly affecting the first phase.

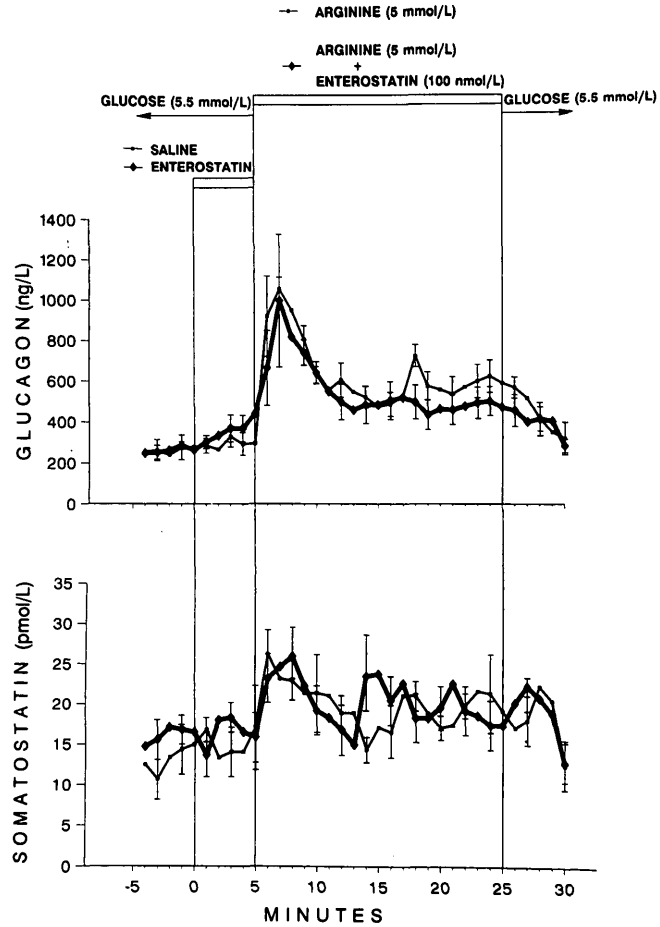

FIG. 2. Effect of 100 mmol/l rat enterostatin on the glucagon and somatostatin responses to 5 mmol/l arginine. Squares correspond to control experiments: from 0 to 5 min, saline infusion; from 5 to 25 min, arginine infusion (n = 6). Rhombi correspond to enterostatin experiments: from 0 to 5 min, enterostatin infusion; from 5 to 25 min, arginine plus enterostatin infusion (n = 6). Data are means ± SE.

As shown in Fig. 1*C*, both phases of the insulin response to 5 mmol/l arginine were inhibited by enterostatin (incremental area: 16.4 ± 5.8 pmol/20 min vs. 49 ± 8.1 pmol/20 min in control experiments, $P < 0.025$).

Figure 2 shows the effect of 100 nmol/l enterostatin on glucagon and somatostatin responses to arginine. The stimulatory effect of 5 mmol/l arginine on glucagon output (incremental area: 14.7 ± 0.8 ng/20 min) was not significantly modified by enterostatin (incremental area: 11.6 ± 2.4 ng/20 min, P = 0.25). Somatostatin levels from control and enterostatin experiments overlapped.

Finally, to explore the functional viability of the β-cell subjected to enterostatin treatment, we examined the insulin response to glucose in the rat pancreas preinfused with a high enterostatin concentration (500 nmol/l) (Fig. 3). As expected, enterostatin abolished the insulin response to an increase in glucose levels from 5.5 to 9 mmol/l glucose (incremental area: 1.5 ± 1.4 pmol/10 min vs. 6.6 ± 1.2 pmol/10 min in control experiments, $P < 0.05$). After a 10-min wash-out period, pancreases preinfused with enterostatin showed an insulin response to a second glucose stimulus comparable to that found in control experiments (incremental area: 4.9 ± 1 pmol/10 min vs. 5.9 ± 2.5 pmol/10 min in control experiments, P = 0.69).

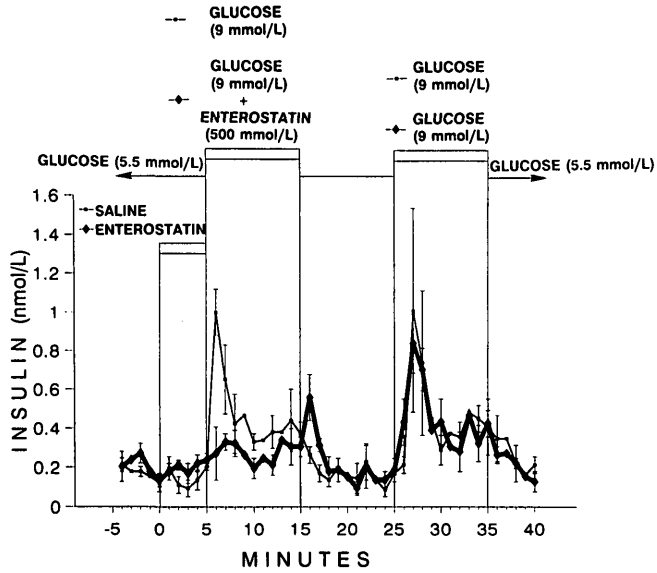

FIG. 3. Effect of previous rat enterostatin (500 nmol/l) infusion on the insulin response to 9 mmol/l glucose in the perfused rat pancreas. Squares correspond to control experiments; from 0 to 5 min, saline infusion; from 5 to 15 min, 9 mmol/l glucose infusion; from 15 to 25 min, wash-out period; and from 25 to 35 min, 9 mmol/l glucose infusion (n = 3). Rhombi correspond to enterostatin experiments: from 0 to 5 min, enterostatin infusion; from 5 to 25, 9 mmol/l glucose plus enterostatin infusion; from 15 to 25 min, wash-out period; and from 25 to 35 min, 9 mmol/l glucose infusion (n = 4). Data are means ± SE.

DISCUSSION

The foregoing results demonstrate that in the perfused rat pancreas, enterostatin inhibits insulin secretion. This inhibition is exerted against various β-cell secretagogues—glucose, arginine, and tolbutamide—that stimulate the release of insulin via different mechanisms (22). The pattern of inhibition, which mainly affects the first insulin secretory phase, was comparable for these three stimuli.

The structure of enterostatin is well preserved across species (23,24). As for the possible physiological role of this peptide, at present, there is very limited information about the presence of enterostatin in systemic blood. It has been hypothesized that after being released from procolipase in the duodenum during digestion, this peptide actively or passively crosses the intestinal brush-border barrier to reach the lymph (25,26). The presence of enterostatin in cat intestinal lymph has recently been reported, and the ratio of the enterostatin concentration in lymph to that in plasma was found to be increased by fat feeding (26). Recently, Bouras et al. (27) have shown that the intestinal brush-border membrane vesicles contain proteases that break down enterostatin, and Huneau et al. (28) have observed that although enterostatin is hydrolyzed with both epithelial sheets and brush-border membranes, there is a small transepithelial passage of immunoreactive enterostatin. Interestingly, intestinal concentrations of enterostatin in rats are in the micromolar range (24) about one order of magnitude higher than that reported for this peptide in human peripheral plasma.

By means of an enzyme-linked immunosorbent assay, Bowyer et al. (29) have shown the presence of enterostatin in the serum of healthy subjects. These authors have reported values ranging from 50 to 100 nmol/l after consumption of a standard meal, and they found that the appearance of enterostatin in urine was concurrent with its elevation in plasma (25). According to this information, the enterostatin concentration used in our perfusions (100 nmol/l) is comparable with that reported for human enterostatin in peripheral blood. Erlanson-Albertsson et al. (14), working in a model of perifused rat islets, observed an inhibition of glucose-stimulated insulin release with an enterostatin concentration of the same order of magnitude (200 nmol/l). However, so far, plasma enterostatin levels have not been measured in rats.

At this stage, conjecture about the mechanism by which enterostatin exerts its insulinostatic effect would be highly speculative. As of now, no specific enterostatin receptors have been identified. Interestingly, the suppressor effect of enterostatin on fat intake in rats can be attenuated by κ-opioid agonists, suggesting that centrally administered enterostatin might act as an opioid antagonist (30). The observation of κ-opiate binding sites in Langerhans islets (31) allows the speculation on the possibility that enterostatin modulates insulin release by inhibiting a κ-opioid pathway within the β-cell.

It should be noted that our observation that preinfusion of the pancreas with a high enterostatin concentration (500 nmol/l) did not alter the insulin response to glucose would exclude a toxic effect of enterostatin on the β-cell.

There is no information about the influence of enterostatin on the other islet hormones. We have found that during perfusion of the rat pancreas with a medium containing 5.5 mmol/l glucose, enterostatin failed to significantly modify glucagon or somatostatin release. Enterostatin was also without effect on the glucagon and somatostatin response to 5 mmol/l arginine. These results seem to indicate that the inhibitory effect of enterostatin on insulin release is not mediated by an α- or δ-cell paracrine effect.

The inhibition of insulin secretion by enterostatin reinforces the parallelism between the anorectic and insulinostatic effects exerted by some peptides, such as amylin and calcitonin (8–13). Indeed, a reduction of caloric intake should be associated with reduced insulin need.

In summary, our results indicate that in the perfused rat pancreas, enterostatin, at putatively physiological concentrations, inhibits insulin secretion without affecting glucagon or somatostatin output. These data point to a role for enterostatin as a regulator of β-cell secretory activity. Because enterostatin is generated in the small intestine after feeding, it might play a role in the enteroinsular axis as an agent with an anti-incretin effect that modulates postprandial hyperinsulinemia.

ACKNOWLEDGMENTS

This work has been supported by FIS grants (94/0282 and 93/0184) from the Ministerio de Sanidad and the Fundación Eugenio Rodríguez Pascual, Spain.

The expert technical assistance of Pilar García and Encarnación Gutiérrez is gratefully acknowledged. We thank Martha Messman for secretarial help.

REFERENCES

1. Erlanson-Albertsson C: The existence of pro-colipase in pancreatic juice. *Biochim Biophys Acta* 666:290–300, 1981
2. Borgström B, Wieloch T, Erlanson-Albertsson C: Evidence for a pancreatic pro-colipase and its activation by trypsin. *FEBS Lett* 108:407–410, 1979
3. Erlanson-Albertsson C: Pancreatic colipase: structural and physiological aspects. *Biochim Biophys Acta* 1125:1–7, 1992
4. Erlanson-Albertsson C, Larsson A: The activation peptide of procolipase decreases food intake in rats. *Regul Pept* 22:325–331, 1988
5. Shargill NS, Tsujii S, Bray GD, Erlanson-Albertsson C: Enterostatin suppresses food intake following injection into the third ventricle of rats.

Brain Res 544:137–140, 1991
6. Okada S, York DA, Bray GA, Erlanson-Albertsson C: Enterostatin (Val-Pro-Asp-Pro-Arg), the activation peptide of procolipase selectively reduces fat intake. *Physiol Behav* 49:1185–1189, 1991
7. Erlanson-Albertsson C, Westrom B, Pierzynowski S, Karlsson S, Ahrén B: Pancreatic procolipase activation peptide-entrostatin-inhibits pancreatic enzyme secretion in the pig. *Pancreas* 6:619–624, 1991
8. Perlow MJ, Freed WJ, Carman JS, Wyatt RJ: Calcitonin reduces feeding in man, monkey and rat. *Biochem Behav* 12:609–614, 1980
9. Morley JE, Levine AS, Broen DM, Hand BS: The effect of calcitonin on food intake in diabetic mice. *Peptides* 3:17–20, 1982
10. Chance WT, Balasubramaniam A, Zhang FS, Wimalawansa SJ, Fischer JE: Anorexia following the intrahypothalamic administration of amylin. *Brain Res* 539:352–354, 1991
11. Morley JE, Flood JF: Amylin decreases food intake in mice. *Peptides* 12:865–869, 1991
12. Silvestre RA, Peiró E, Dégano P, Miralles P, Marco J: Inhibitory effect of rat amylin on the insulin responses to glucose and arginine in the perfused rat pancreas. *Regul Pept* 31:23–31, 1990
13. Alwmark A, Stavinoha MW, Cooper CW, Greeley GH, Thompson JC: Calcitonin inhibition of insulin release from isolated rat pancreatic islets. *Diabetes* 35:58–60, 1986
14. Erlanson-Albertsson C, Hering B, Bretzel RG, Federlin K: Enterostatin inhibits insulin secretion from isolated perifused rat islets. *Acta Diabetol* 31:160–163, 1994
15. Leclercq-Meyer V, Marchand J, Leclercq R, Malaisse WJ: Glucagon and insulin release by the in vitro perfused rat pancreas. *Diabete Metab* 2:57–65, 1976
16. Silvestre RA, Miralles P, Moreno P, Villanueva ML, Marco J: Somatostatin, insulin and glucagon secretion by the perfused rat pancreas from the cysteamine-treated rats. *Biochem Biophys Res Commun* 134:1291–1297, 1986
17. Yalow RS, Bergson SA: Immunoassay of endogenous plasma insulin in man. *J Clin Invest* 39:1157–1175, 1960
18. Herbert V, Lau KS, Gottlieb CW, Bleicher SJ: Coated charcoal immunoassay of insulin. *J Clin Endocrinol Metab* 25:1375–1384, 1965
19. Faloona GR, Unger R: Glucagon. In *Methods of Hormone Radioimmunoassay*. Jaffe BM, Behrman HR, Eds. New York, New York Academic, 1974, p. 317–330
20. Harris V, Conlon JM, Srikant CB, McCorkle K, Schusdziarra V, Ipp E, Unger RH: Measurement of somatostatin-like immunoreactivity in plasma. *Clin Chim Acta* 87:275–283, 1978
21. Siegel S: *Non Parametric Statistic for the Behavioral Changes*. New York, McGraw-Hill, 1978
22. Flatt PR: *Nutrient Regulation of Insulin Secretion*. London, Portland Press, 1992 (monogr. no. 1)
23. Wicker C, Puigserver A: Rat pancreatic colipase mRNA: nucleotide sequence of a cDNA clone and nutritional regulation by a lipidic diet. *Biochem Biophys Res Commun* 167:130–136, 1989
24. Mei J, Bowyer RC, Jehanli AM, Patel G, Erlanson-Albertsson C: Identification of enterostatin, the pancreatic procolipase activation peptide in the intestine of rat: effect of CCK-8 and high-fat feeding. *Pancreas* 8:488–493, 1993
25. Bowyer RC, Rowston WM, Jehanli AMT, Lacey JH, Hermon-Taylor J: Effect of a satiating meal on the concentration of procolipase propeptide in the serum and urine of normal and morbidly subjects. *Gut* 34:1520–1525, 1993
26. Townsley C, Erlanson-Albertsson C, Ohlsson A, Reed RK: Enterostatin efflux in cat intestinal lymph: relation to lymph flow, hyaluronan and fat absorption (Abstract). *Exp Biol* 9:AA367, 1995
27. Bouras M, Huneau JF, Luengo C, Erlanson-Albertsson C, Tome D: Metabolism of enterostatin in rat intestine, brain membranes, and serum: differential involvement of proline-specific peptidases. *Peptides* 16:399–405, 1995
28. Huneau JF, Erlanson-Albertsson C, Beauvallet C, Tome D: The in vitro intestinal absortion of enterostatin is limited by brush-border membranes peptidases. *Regul Pept* 54:495–503, 1994
29. Bowyer RC, Jehanli AMT, Patel G, Hermon-Taylor J: Development of enzyme-liked immunosorbent assay for free human pro-colipase activation peptide (APGPR). *Clin Chim Acta* 200:137–152, 1991
30. Barton C, York DA, Bray GA: Kappa-opioids and enterostatin differentially influence high fat diet selection and consumption (Abstract). *Exp Biol* 9:A1005, 1995
31. Khawaja XZ, Green IC, Thorpe JR, Titheradge MA: The occurrence and receptor specificity of endogenous opioid peptides within the pancreas and liver of the rat. *Biochem J* 267:233–240, 1990