Structure-function studies on enterostatin inhibition of insulin release

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Abstract

The pentapeptide enterostatin (Val-Pro-Asp-Pro-Arg or VPDPR[1]) has been shown to inhibit insulin secretion both *in vivo* and in isolated rat islets. We report on structure-function studies of enterostatin in rat islets and in RINm5F insulinoma cells. In isolated rat islets, the tyrosinated version of enterostatin (YVPDPR) had ~10 fold reduced potency at lower concentrations (10nM *versus* 1nM) in inhibiting insulin release compared to nascent enterostatin and did not exhibit activity at 1μM concentrations. Replacement of the L-valine with D-valine caused total loss of biological activity. The peptide YPDPR was more efficacious than native enterostatin at 0.1nM but lost activity at higher concentrations. However, in RINm5F cells, the inhibitory effect of YPDPR was preserved. 3-iodo-YPDPR exhibited a similar inhibitory profile to enterostatin across a wide concentration. These studies demonstrate the functional importance of the N-terminal sequences of enterostatin in regulating insulin secretion but also suggest tolerance to some modifications. Radio-iodinated-YPDPR may be useful in characterising putative enterostatin receptors.

Keywords : *Enterostatin, Enterostatin Analogues, Insulin Secretion, Rat Islets, RINm5F cell-line*

Introduction

The peptide enterostatin is generated via proteolytic cleavage of pancreatic procolipase in the intestinal lumen.[1,2] Whilst pancreatic colipase acts as an essential cofactor in the hydrolysis and subsequent absorption of triglycerides, enterostatin may act as a feedback signal to inhibit fat consumption.[3-5] Food intake, in particular dietary fat, stimulates the release of procolipase and presumably also of enterostatin.[2,6] Peripheral[3] as well as central[7,8] administration of enterostatin have been shown to inhibit food-intake, particularly of fatty foods, and chronic intracerebroventricular infusion of enterostatin in the rat reduces food-intake,[7,8] fat deposition and body weight gain.[7]

In addition to the central and vagally-mediated actions of enterostatin as an inhibitor of fat-intake, this peptide exerts a direct inhibitory effect on the endocrine pancreas, resulting in the inhibition of insulin release from islets [9-11] and perfused pancreas, [12,13] without affecting glucagon or somatostatin release.[13] The inhibitory effect of enterostatin on insulin release appears to be mediated at least in part via suppression of cAMP accumulation.[11]

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Consistent with this hypothesis was the recently reported inhibition of insulin release by enterostatin evoked by glucagon-like peptide-1 and gastric inhibitory peptide, both of which elevate cAMP levels.[12] In contrast, enterostatin has no effect on insulin secretion in response to agents that activate the phospholipase C pathway, e.g. cholecystokinin.[12] Despite these advances in understanding the mechanisms of enterostatin inhibition of insulin release, the molecular nature of putative enterostatin receptors is unknown and enterostatin receptors remain uncharacterised, in part due to the lack of suitable high affinity ligands.

The structure of enterostatin is fairly well conserved across different species. Based on colipase gene sequence as well as amino acid analysis three enterostatin sequences have been described to date: VPDPR,[4] APGPR[6,14] and VPGPR.[15] Structure-function studies on central actions of enterostatin and its analogues indicate that several substitutions and deletions are tolerated.[5] For example, 1nmol YPDPR or PDP were as potent as 1nmol VPDPR in inhibiting high-fat food intake.[5] However, PDP had no effect on insulin secretion, raising the possibility that different receptors may be involved in mediating enterostatin actions in the brain (to regulate feeding) versus periphery (to regulate insulin secretion). The C-terminally truncated enterostatin peptide, VPDP was found to produce a potent inhibitory effect on food-intake, as well as on insulin release in vitro[9] but no effect on plasma insulin was seen when VPDP was infused intraduodenally.[16] Thus, the structural requirements for enterostatin inhibition of insulin release are incompletely understood.

The aim of the present study was to elucidate in more detail the necessary structural features for enterostatin's inhibitory effect on insulin release. We report that although some modifications in the N-terminus of VPDPR abrogate biological function, other modifications result in retention of biological activity. Notably, the enterostatin derivative 3-iodo-YPDPR retains almost full activity and could be useful for the pharmacological characterisation of putative enterostatin receptors in islets and other tissues.

Materials and Methods

Peptide synthesis and iodination

Peptides were synthesised by solid-phase chemistry and were purified by HPLC and estimated >99% purity. The peptide Tyr-Pro-Asp-Pro-Arg (YPDPR) was iodinated using a sodium iodide/lactoperoxidase/glucose oxidase protocol, purified by HPLC using a Waters Symmetry C18 column. This was carried out at ambient temperature using mobile phases composed of solution A (water/acetonitrile 95:5 v/v + 0.1% Trifluoroacetic acid - TFA), solution B (water/acetonitrile 85:15v/v + 0.1% TFA) and solution C (water/acetonitrile 10:90v/v + 0.1% TFA). Elution was achieved using linear gradients at a flow rate of 1ml/min: 0-100% solution B over 30 min, then 100%B to 100%C over 5 min and hold at 100%C for 10 min. Retention time was approximately 27 min and detection of radioiodinated peptide was achieved with UV spectroscopy at 220 nm coupled with gamma-detection.

Product identification was demonstrated by co-elution with unlabelled standard and Liquid Chromatography-Mass Spectroscopy

(LC-MS) was used on unlabelled runs to identify the product of reaction on similar scale to the "hot" run. LC-MS shows M+ for peak at ca. 27 min as 773.1, corresponding to the mono-iodination of starting material. Although this does not prove iodination at 3 position, the chemistry of the reaction is considered to dramatically favour iodination in this position.

Isolation of pancreatic islets

Islets were isolated from 8-12 weeks old SD rats using bile ductal infusion and static digestion at 37^{o}C for 40 min with 0.6 mg/ml collagenase (Type 4, Worthington Biochemical, 0.6mg/ml) in 10 mM HEPES buffered Hank's balanced salt solution (HBSS) containing 0.2% bovine serum albumin (BSA). The digest was processed through a Ficol gradient (25-23-20.5-11%) in HBSS to yield purified islets. Isolated islets were cultured in minimum essential medium (MEM) supplemented with 10% calf serum for 6 h at 37^{o}C in 5% CO_2 incubator. Islets (diameter 100-180 μm) were randomly selected after a one-hour pretreatment with different concentrations of enterostatin, its analogues or vehicle in RPMI-1640 medium containing 3.3 mM glucose. Two islets from each pretreatment group were transferred into fresh tubes containing 200μl of medium supplemented with 16.7 mM glucose and the same concentration of enterostatin, its analogues or vehicle. The incubations were continued for another 30 min in a shaking water bath maintained at 37^{o}C supplied with 95% O_2:5% CO_2. The incubation medium was removed and kept frozen at -20^{o}C for subsequent insulin assay using specific radioimmunoassay supplied by Linco Research Labs.

RINm5F cell culture

RINm5F cells were maintained in RPMI1640 medium, containing 11 mM glucose and supplemented with 10% foetal calf serum and 1% glutamine. Cells were seeded in 48-well plates 2-3 days prior to performing insulin secretion studies. Insulin secretion studies were performed by washing the cells once in Krebs/Ringer bicarbonate buffer, previously gassed and supplemented with 0.1% BSA, 1 mM $CaCl_2$ and 11 mM glucose. Cells were then incubated for 2 h in the same buffer. The medium was replaced with fresh buffer containing enterostatin for 30 min prior to addition of insulin secretagogue (arginine or glibenclamide). Cells were incubated for a further 30 min, after which the medium was sampled and frozen at 20^{o}C for subsequent insulin radioimmunoassay.

Data and Statistical Analysis

Data are presented as mean $\pm$ SEM. Insulin secretion for different treatments were calculated as ng insulin secreted per islet per 30 minutes or as ng/ml for RINm5F cells. Data were pooled together from the same treatment group. Statistical tests for difference between the mean of treatment groups were performed using one-way ANOVA in conjunction with Student's t-test, $p<0.05$ representing a significant difference.

Results and Discussion

Enterostatin and enterostatin analogues

Previous studies have shown that the C-terminal arginine is not required for the action of VPDPR in islets.[9] To investigate the importance of the N-terminal part of enterostatin and to

Table 1: Synthetic enterostatin peptides used in insulin secretion studies.

Peptide	Peptide Sequence	Single-letter code
1	Val-Pro-Asp-Pro-Arg	VPDPR
2	D-Val-Pro-Asp-Pro-Arg	D-VPDPR
3	Tyr-Val-Pro-Asp-Pro-Arg	YVPDPR
4	Tyr-Pro-Asp-Pro-Arg	YPDPR
5	3-[Iodo]-Tyr-Pro-Asp-Pro-Arg	3-I-YPDPR
6	Arg-Pro-Pro-Asp-Val	RPPDV

Table 2: Effect of various enterostatin analogues on insulin release in rat islets or in RINm5F insulinoma cells. Values are expressed as % of secretion in response to 16.7mM glucose in islets, or in response to 20mM arginine in RINm5F cells, these being set at 100%. (n=6). N.D.=not determined, * $p<0.05$ and ** $p<0.01$ vs. control incubations in the absence of peptide.

		Isolated islets		RINm5F cells	
Peptide Conc.	**VPDPR**	**YVPDPR**	**YPDPR**	**VPDPR**	**YPDPR**
0	100%	100%	100%	100%	100%
10^{-10} M	82±7.4 *	N.D.	38±7.0 *	84±2.3*	82±4.4 *
10^{-9} M	89±2.05*	98±10.0	N.D.	72±3.8*	85±10.2
10^{-8} M	81±5.3 **	72±6.8 **	67±9.2	76±2.3*	70±7.2 **
10^{-7} M	62±6.8 **	61±6.1 **	N.D.	76±4.2*	81±5.7 *
10^{-6} M	69±4.1 **	96±10.9	83±23	75±5*	86±5.8
10^{-5} M	42±6.5 **	N.D.	N.D.	N.D.	N.D.

ascertain whether active tyrosinated enterostatin derivatives can be identified, several versions of the peptide were synthesized (Table 1) and their effects were tested on glucose-induced insulin secretion in isolated rat islets.

Inhibition of insulin release by enterostatins in islets and in RINm5F insulinoma cells

The present study confirms previous reports on inhibition of insulin release by VPDPR in islets and extends this observation to RINm5F cells (Table 2). The effect of VPDPR is specific and dependent on the maintenance of certain structural features of the pentapeptide, since the synthetic peptide, RPPDV, which consists of the same amino acids that comprise enterostatin, had no effect on insulin release. Inhibition of high-fat food intake in rats has been observed with all three enterostatins although VPGPR had the lowest potency.[5] In contrast, VPGPR has been reported to be ineffective in influencing insulin release.[16] The divergence in the effect on insulin secretion by various enterostatins versus their feeding effects, may indicate the existence of different enterostatin receptors.

Recent studies have shown a direct inhibitory effect of enterostatin on insulin release in isolated rat islets in response to nutrient secretagogues (glucose, arginine) and a number of pharmacological agents (sulphonylureas, kappa-opioid receptor ligands or PKC activators). These data indicate that enterostatin modulates insulin release in response to a variety of agents that affect first phase as well

as the second phase of insulin release.[11] In order to determine the suitability of an insulinoma line for characterisation of enterostatin receptors the responsiveness of RINm5F cells was assessed. In this cell-line, VPDPR inhibited insulin release in response to arginine (Table 1) and glibenclamide. However, no evidence was found for inhibition of cAMP levels by enterostatin in RINm5F cells in response to a variety of agonists, including forskolin, glucagon-like peptide 1 or the kappa-opioid receptor agonist, U50,488 (not shown). The lack of effect of enterostatin on cAMP levels in RINm5F cells, despite inhibition of insulin release, suggests that mechanisms in addition to cAMP generation may mediate enterostatin action, at least in this cell-line. In rat islets, the inhibitory effects of enterostatin were reported to be prevented by the cAMP analogue, 8-Br-cAMP, suggesting that enterostatin's actions are mediated, at least in part, via actions on cAMP levels.[11] In addition, the stimulatory effect of U50,488 on cAMP generation in rat islets was abolished by 100 nM enterostatin.[11]

The inhibition of insulin release in islets by enterostatin occurred with nanomolar potency and was generally maintained at higher concentrations. In some experiments the concentration-response curve followed a U-shaped curve. A similar concentration-response curve has been reported for effects of enterostatin on inhibition of fat-intake, where only intermediate concentrations of enterostatin were effective. The basis for the U-shaped dose-response curve has been proposed to arise from the presence of high and low affinity binding sites for the peptide.[5] Alternatively, high concentrations of enterostatin may lead to receptor down-regulation. It is interesting to note that in taste cells enterostatin inhibits ATP-sensitive K^+ channels.[17] If this occurred in beta-cells, enterostatin would be expected to stimulate insulin release, but this was not observed under any circumstance. Although we did not study K^+ channel activity in the present study, it may be speculated that at high concentrations enterostatin may affect K^+channels in islets and this may contribute to the U-shaped dose-response curve. Identification of enterostatin receptors and measurement of K^+channel activity in islet beta-cells will be required to address these points.

Effect of enterostatin analogues on insulin release

The importance of the N-terminal residue of enterostatin was investigated by replacing the L-valine with (i) D-valine, (ii) L-tyrosine or (iii) addition of an L-tyrosine to generate the hexapeptide, YVPDPR. Replacement of the N-terminal L-valine residue with D-valine abrogated the insulin inhibitory activity of the peptide. This demonstrates that the N-terminal valine may be necessary for biological function and is consistent with a previous report showing a lack of effect of D-VPDPR on high-fat food consumption.[5] However, certain changes in the N-terminal structure, for example, replacement of valine with alanine, may maintain biological activity- the naturally occurring enterostatin APGPR inhibits feeding,[5] although another report found no effect of APGPR on food intake in the rat,[18] and des-argVPDP has been shown to inhibit insulin release.[9] It has been found that APGPR also inhibits insulin release in rat islets (York and Liou

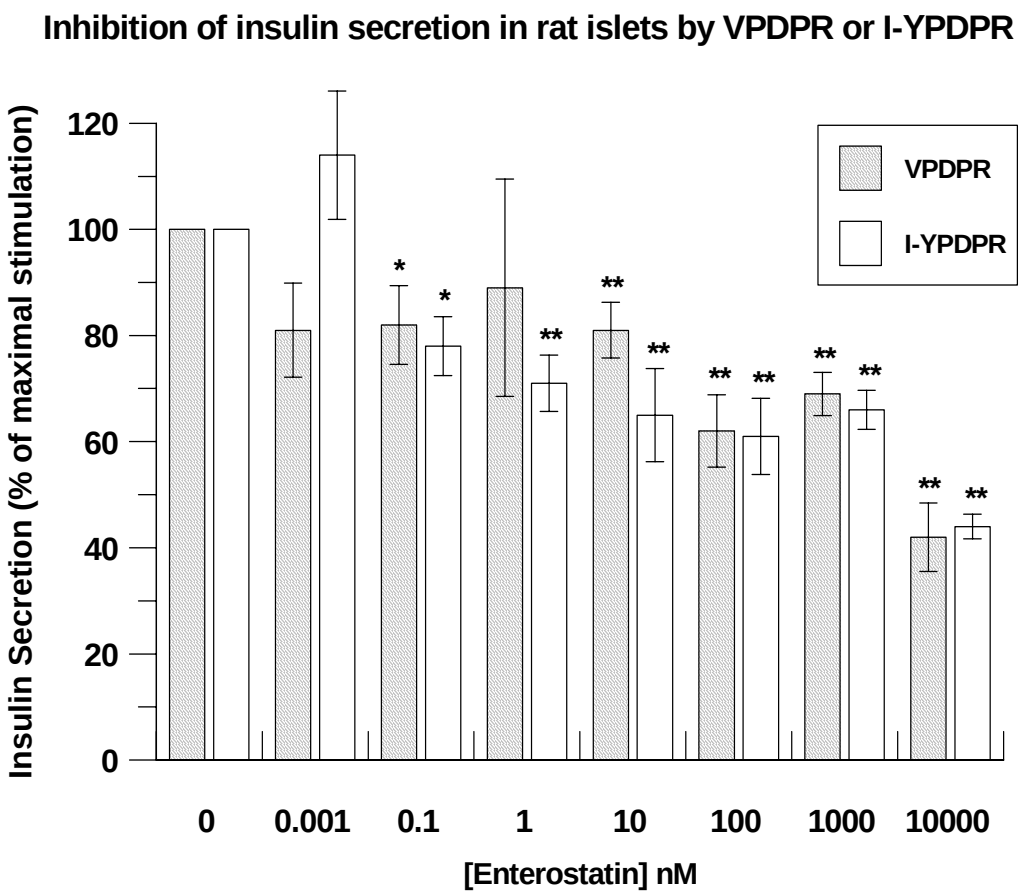

Figure 1: Comparison between enterostatin (VPDPR) and the iodinated enterostatin derivative, I-YPDPR, on 16.7mM glucose stimulation of insulin release in rat islets. Insulin secretion in response to 16.7mM glucose was 1.35±0.15 ng/islet (n=12).
Data are mean±SEM, *p<0.05, **p<0.005 vs control.

unpublished data).

The presence of the amino acid tyrosine renders peptides suitable for radio-iodination. Such radio-iodinated peptides may be useful for ligand binding analyses of the peptide receptor, provided that the iodinated peptide maintains a high affinity for that receptor.

We therefore synthesised and tested the effect of YVPDPR. Compared to VPDPR, YVPDPR-inhibition of glucose-induced release occurred only within a very narrow range (Table 2), suggesting that this peptide may not be suitable for ligand binding studies.

Previous studies have shown that 1 nmol YPDPR is equipotent to VPDPR in inhibiting food intake, indicating that this modification in the N-terminal residue is tolerated.[6] In order to test whether a similar structure-activity relationship may exist for effects on insulin release, we used a wide range of concentrations of YPDPR. YPDPR inhibited insulin release in both islets and RINm5F cells with a potency at least as high as that observed with natural enterostatin VPDPR (Table 2). However, higher concentrations of YPDPR were less effective in inhibiting insulin release, in keeping with data from some experiments with VPDPR, which show a U-shaped concentration-response (see above).

Nonetheless, the high potency of YPDPR as an inhibitor of insulin release suggests that this peptide may be suitable for (radio)iodination and could be a useful ligand for the characterisation of putative enterostatin receptors. As a prelude to these studies, YPDPR was cold-iodinated and the purified 3-[I]-YPDPR was assessed for its ability to inhibit insulin release from rat islets (Figure 1). 3-[I]-YPDPR turned out to be as potent and as efficiacious as VPDPR (Figure 1), suggesting that the iodinated peptide may be suitable for characterising enterostatin receptors in insulin-secreting cells and other enterostatin-responsive tissues. Since the membrane yield from islets is limiting and we had shown responsiveness of RINm5F cells to VPDPR, 3-[^{125}-I]-YPDPR was employed in competition ligand binding studies using membranes prepared from RINm5F cells. Total binding to RINm5F membranes was low and was not competed by VPDPR, APGPR or 3-[I]-YPDPR; i.e. no specific binding could be detected. Under similar conditions we did detect, as expected, specific binding of glucagon-like peptide-1. Lack of specific binding by the enterostatin analogue 3-[I]-YPDPR, despite its potent functional effect on insulin release (Figure 1), may be due to an expression level of putative enterostatin receptors below the detection limit of ligand binding assays. Other tissues, for example islets or hypothalamus, are alternative sources of membranes for binding studies using 3-[I]-YPDPR and should be the focus for future work in this area.

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