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Cleavage of obestatin by DPP-IV and inhibition of incretin effect on MIN6 cells

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Abstract: *Obestatin is a poorly characterized peptide secreted from gastrointestinal tract (GIT) and pancreatic islet. It is a 23-amino acid amidated peptide hormone formed through posttranscriptional processing of ghrelin gene. Some studies suggest that obestatin plays an important role in gluco-regulation.These features of obestatin are similar to glucagon-like peptide-1 (GLP-1), which is also synthesized and secreted from GIT and formed through the posttranslational processing of proglucagon gene.Active form of GLP-1 potentiates insulin secretion mainly in presence of food or glucose stimuli (incretin effect), but it is inactivated by the cleavage of dipeptidyl peptidase-IV (DPP- IV) enzyme. Obestatin was evaluated on MIN6 mouse pancreatic β-cells for incretin effect and survival. Cleavage and inactivation of obestatin by DPP-IV was confirmed by mass spectrometric analysis. Comparisons were made with standard GLP-1.In response to the incubation with external obestatin, endogenous secretion of obestatin was diminished, DPP-IV enzyme was found to be inhibitory on incretin effect. Mass spectrometric analysis of the incubated mixture of obestatin and DPP-IV revealed cleavage of obestatin .XTT assay and media concentration of DNA revealed positive influence of obestatin on MIN6 cells survival or proliferation. We conclude that obestatin exert incretin effect and positively influence survival of pancreatic cells similar to GLP-1.Obestatin was also found to be cleaved by DPP-IV enzyme at amidated C-terminal.*

Keywords: *DPP-IV, Ghrelin, GIT, GLP-1, Obestatin.*

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I. Introduction

Gluocagon-like peptide-1 (GLP-1) is one of the several products of posttranslational processing of proglucagon gene transcript in intestinal L-cells of gastrointestinal tract (GIT)[1]. This category of peptides are known as incretins, because they potentiate insulin secretion in response to food or glucose stimuli; known as incretin effect. Some other poorly characterized peptides also exert similar functions and known as glucose dependent insulinotropic peptides (GIPs) [1-3]. GLP-1 and GIPs are very important peptides to treat diabetes due to their pleiotropic effects such as stimulation of insulin secretion, diminish glucagon secretion, delayed gastric emptying, reduced hepatic glucose output and increased pancreatic β-cell survival [4]. However, cleavage of GLP-1 through dipeptidyl peptidase-IV(DPP-IV) enzyme converted it into inactive form. DPP-IV enzyme is a subset of oligopeptidase encoded by CD26. It is widely distributed in various tissues. This enzyme cleaves off N-terminal amino acids and inactivates number of biologically active peptides including GLP-1 and GIPs. This enzyme specifically cleaves off dipeptides from protein having alanine or proline at position 2 of their N-terminal [5]. Similar to GLP-1, obestatin is also a 23-amino acid amidated peptide hormone, identified as a product of ghrelin gene (GHRL). The m-RNA of GHRL gene has four exons and through post transcriptional modifications it encoded five products of similar structures. Further, posttranslational cleavage of one product C-ghrelin (unacylated) is presumed to form obestatin *in vivo* [6].Biological functions of obestatin are still poorly understood but it is thought to be anorectic peptide which decreases food intake. However number of other functions including promotion of insulin secretion, survival of β-cells and pancreatic islets, decrease in weight gain, gastric emptying and jejuna motility being suggested, but mechanisms are not well understood [6-8]. Some reports also suggested that obestatin functions through G-protein coupled receptor (GPR39), which is also debatable and receptor for obestatin remains unknown [8-10].After careful review of the functions, receptor studies and amino acid sequence of human obestatin, we hypothesized that obestatin may exert incretin effect and may be cleaved by DPP-IV at its C-terminal because this is amidated and having alanine at position 2. Therefore, in present study, we addressed two major questions whether obestatin functions similar to GLP-1 and whether DPP-IV cleaves off obestatin into its inactive form.

II. Materials and methods

2.1 Chemicals

Human obestatin, Gly-pro-p-nitroanilide, Dipeptidyl peptidase-IV (DPP-IV), GLP1RA (GLP-1 non peptide receptor agonist) and standard GLP-1 were purchased from Sigma-aldrich.MIN6 cells were gifted by Dr. Vasudevan Sheshadri, Scientist E, NCCS, Pune, India. Media DMEM was purchased from Hi-media Pvt. Ltd, India. Insulin and obestatin estimations were done using ELISA kits from IRI, USA. XTT kit was purchased from Hi-Media Pvt., Ltd. India.

2.2Methods

2.2.1Studies on MIN6 cells and mass spectrometric analysis

MIN6 cells were cultured in DMEM initially for 5 days. Then three different concentrations (10nM, 5nM and 01 nM) of GLP-1 standard and obestatin were used in preliminary experiment. Out of these concentrations 1 nM concentration of obestatin was found suitable for insulin secretion from MIN6 cells. Therefore, all the experiments conducted at 1nM concentration of these two peptides. In each setup 20 µl of GLP-1 or obestatin was used in 200 µl of media. The incubation time for each experiment was set for 48 hrs. DPP-IV concentration was 0.05 U/ ml and 10 µl in each well it was added and incubated for 48 hrs. Media was containing 25 mM glucose [10]. Insulin and obestatin estimations in media samples were conducted by ELISA kits. Estimation of XTT was carried out using kit method. XTT was done post experimental incubation of 48 hours of cells with XTT substrate by following the protocol provided by manufacturer. DNA estimation was done using spectrophotometric analysis at 260 and 280 nm. Absorbance at 320 nm was also used to subtract from the absorbance of 260 nm to base line turbidometric errors. After calculation of DNA purity quantitation was done. Mass spectral data were obtained by recording the positive mode electro spray ionization (ESI) on a Brukermicr OTOF-Q II mass spectrometer[12].

In silico, molecular docking study was conducted using AutoDock4.0, where as to model protein structures, modeller 9.0 was used. To visualize these structures PyMol was used. These all tools are freely available online. It is noteworthy that to predict the natural way of interactions of GLP-1 and obestatin with GLP-1R (GLP-1 receptor) and DPP-IV free or blind docking was considered, as reported elsewhere[11, 13-15].

III. Results

3.1. Effect of obestatin and GLP-1 on insulin secretion, concentrations of obestatin, XTT and DNA in media from MIN6 cells (Table 1)

In this study a profound increase was observed in insulin secretion in response to administration of obestatin and standard GLP-1. The increase in insulin concentration was approximately three folds greater than control values in both the test peptides. However, in presence of external GLP-1 and obestatin into the media the overall concentration of obestatin was decreased. On the parameter of cell survival, an increase was observed in the absorbance of XTT in both GLP-1 and obestatin treated groups. In obestatin treated group, no significant change was observed in DNA concentration in media, while significant decrease was observed in GLP-1 treated group.

3.2. Effect of obestatin and GLP-1 on insulin secretion, obestatin, XTT and DNA in media from MIN6 cells in presence of DPP-IV (0.05U/ml) (Table 2)

In this study, presence of DPP-IV, inhibited insulin secretion from MIN 6 cells. The most pronounced effect was observed in DPP-IV control where more than 50% decrease was observed. However, a significant increase was observed in response to obestatin and GLP-1 treated groups. No significant change was found in XTT values of DPP-IV group as compared to control. However, almost four fold increase was observed in XTT values of GLP-1 treated group, whereas three folds higher in obestatin treated group. In case of DNA concentration, it was found to be decreased only in GLP-1 treated group.

3.3 Mass spectrometric analysis of DPP-IV and obestatin (Fig 1)

Mass spectrometric analysis revealed the release of a dipeptide containing alanine-leucine from obestatin, in response to incubation of obestatin along with DPP-IV enzyme.

4. Comparative study using in silico molecular docking of GLP-1 and obestatin with DPP-IV and GLP-1Recpetor (Fig 2 and 3)

We observed that both GLP-1 and obestatin interact and bind with the active cavity of DPP-IV and GLP-1 receptor; however the strength of binding was higher in case of GLP-1.

Table 1: Effects of obestatin and GLP-1 on insulin, obestatin, XTT and DNA concentration in media from MIN6 cells

Groups	Insulin (µU/ml)	Obestatin (pg/ml)	XTT(Abs)	DNA(µg/ml)
Control	10.52 ± 0.420	8.77 ± 0.175	0.216 ± 0.013	43.41 ± 0.546
GLP-1	31.08 ± 2.40[a]	6.31 ± 0.186[a]	2.682 ± 1.062[c]	32.086 ± 3.17[b]
Obestatin	31.02 ± 1.160[a]	5.40 ±0.429[a]	2.008 ± 0.198[c]	42.79 ± 2.039[c]

Data are means ±S.E.M.(n=3);[a],$P<0.001$;[b],$P<0.01$and[c],$P<0.05$whencomparedwith the values of control.

Table 2: Effects of obestatin and GLP-1 on insulin, obestatin, XTT and DNA concentration in media from MIN6 cells in presence of DPP-IV

Groups	Insulin (µU/ml)	Obestatin (pg/ml)	XTT(Abs)	DNA(µg/ml)
Ctrl	9.52 ± 0.080	8.58 ± 0.897	0.233 ± 0.015	43.89 ± 2.263
DPP-IV	4.286 ±0.053[b]	5.95 ± 0.790[c]	0.386 ±0.073[c]	41.11 ± 0.071[c]
GLP-1+DPP-IV	7.413 ±0.802[z]	5.50 ± 0.529[z]	1.314 ±0.359[x]	29.125±1.267[x]
Obestatin +DPP-IV	8.850 ±1.245[y]	6.49 ± 0.693[z]	0.826 ±0.136[z]	41.22 ±0.596[z]

Data are means ±S.E.M.(n=3);[a],$P<0.001$;[b],$P<0.01$and[c],$P<0.05$whencomparedwith the values of control and[x],$P<0.001$;[y],$P<0.01$ and[z],$P<0.05$ when compared with the respective values of DPP-IV control.

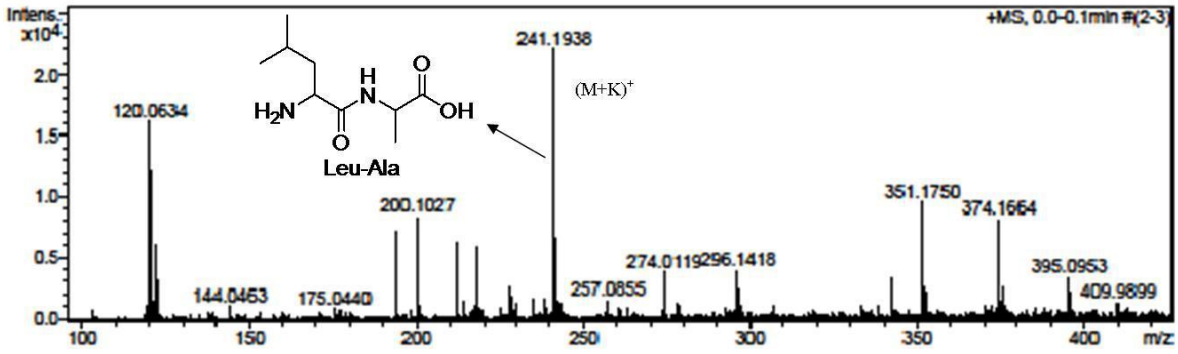
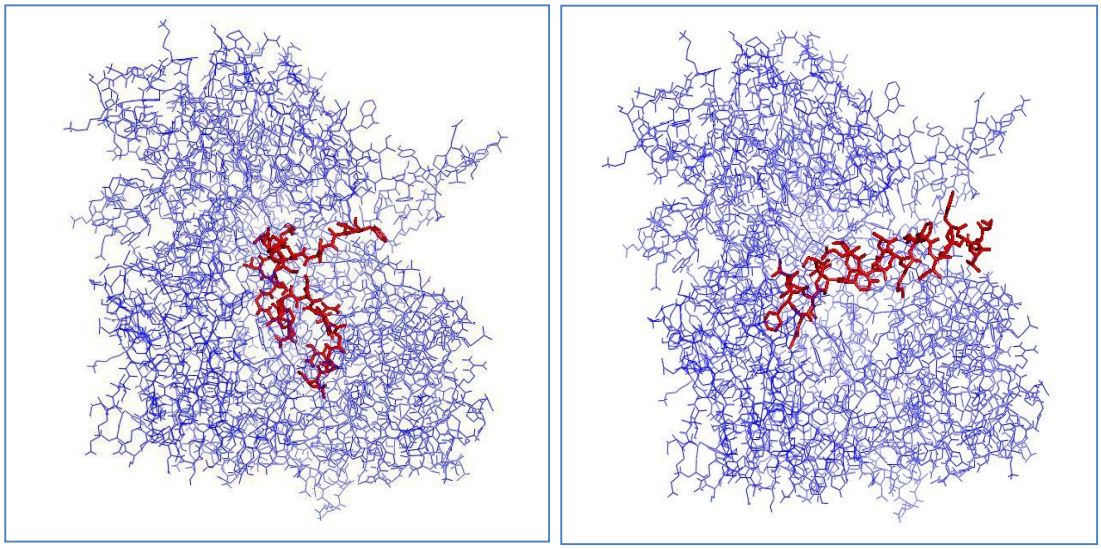


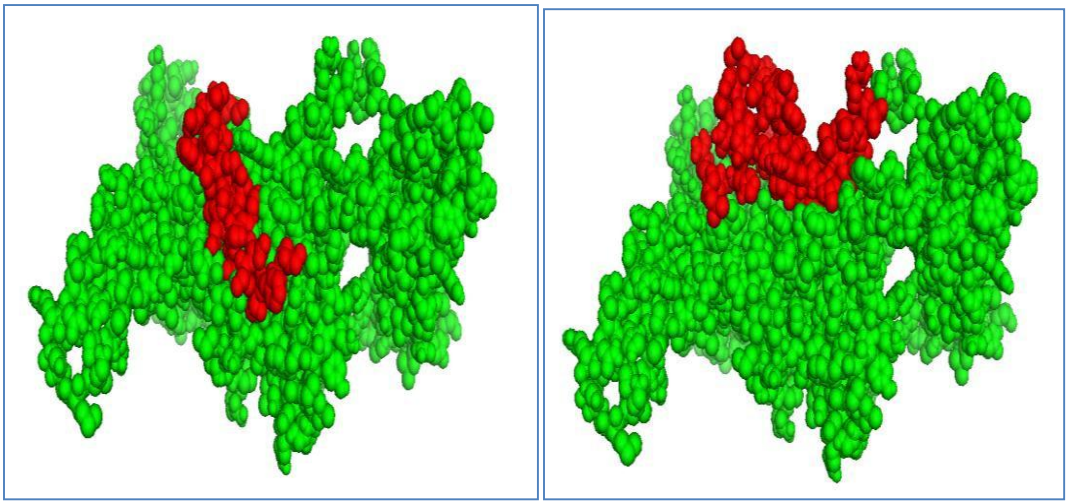
Fig.l. Mass spectrometry shows the potassium adduct peak of dipeptide Leu-Ala, released from C-terminal of human obestatin in presence of DPP-IV



(1)DPP-IV with obestatin (2) DPP-IV with GLP-1

Fig.2: *In silico* molecular docking of GLP-1 and obestatin with DPP-IV

Parameters	DPP with Obestatin	DPP with GLP-1
Binding Energy	-5.7	-4.56
Ligand Efficiency	-0.08	-0.02
Hydrogen Bonds	1 (Ser473:HG)	3 (Arg471:HH12; Ser473:HG; Arg560:HH12)



(1)GLP1-R and GLP-1 (2) GLP-1R and obestatin

Fig. 3:*In silico* molecular dockingofGLP-1 and obestatin with GLP-1R

	GLP-1R with GLP1	GLP-1Rwith Obestatin
Binding Energy	-5.63	1.23
Ligand Efficiency	-0.23	-0.10
Hydrogen Bonds	3 (GLN 45:HE22; ARG299:HH11; ASN300:HD22)	1 (ARG 299:HH11)

IV. Discussion

Data from **table 1** consistently revealed that obestatin stimulated insulin secretion from MIN6 cells, similar to GLP-1. It is noteworthy that GLP-1 or GIPs increase insulin secretion mainly in presence of glucose or food stimuli [5, 6]. In case of GLP-1 potentiating the insulin secretion is mainly mediated through GLP-1 receptor; while in case of obestatin receptor is not known, but due to observed functional similarity, we hypothesize that obestatin also work through GLP-1 receptor [1]. In GLP-1 treated group, increase in absorbance of XTT along with the reduction in DNA concentration strongly suggest the positive influence on pancreatic cell survival and proliferation which is also in agreement with earlier reports [5, 6].However, in case of obestatin only XTT was increased without any significant change in DNA concentration, suggesting that it might be involved only in cell proliferation, but did not alter cell survival. It is noteworthy that XTT assay and DNA concentration in media were quantified 48 hours post experimentation, in this duration some cells may undergo apoptosis and some may proliferate. It is well documented that increase in XTT activity is associated with the presence of healthy cells, which may be due to increased survival and / or proliferation, while DNA in media represents late phases of apoptotic or necrotic cells. Therefore, increased XTT without any change in DNA concentration are more likely when proliferation increases without any change in survival of MIN6 cells. It is also in agreement with earlier reports on obestatin and GLP-1 [1, 4, 7]. It is imperative to mention here that obestatin is known to increase beta cell proliferation and insulin secretion might be through GLP-1receptor [7]. In this pathway, obestatin stimulate transcription of GLP-1R, which increases level of cAMP that result blockade of K+ channel and opening of Ca^{+2} release which ultimately promote insulin secretion [7,16-18]. Another report on obestatin suggests proliferation of pancreatic cells via activation of PI3-kinase pathway [7]. We also observed obestatin secretion from MIN6 cells, which is also in accordance with earlier reports where synthesis and release of ghrelin and obestatin from pancreatic islet was shown [19, 20]. In present study, MIN6 cells showed secretion of obestatin in presence of 25 mM glucose (present in media), but if GLP-1 or obestatin itself was abundant outside the cells then synthesis and/or secretion of endogenous obestatin might be diminished. This effect might be either due to decreased synthesis and/or secretion of obestatin from MIN6 cells, which could not be ruled out.

Data from **table 2**, where presence of DPP-IV decreased insulin secretion, while in presence of DPP-IV along with GLP-1 or obestatin increased insulin secretion, as compared to DPP-IV control. This clearly established that obestatin exert incretin effect, while presence of DPP-IV inhibited the same. Another important finding was decreased concentration of obestatin in DPP-IV treated group, which confirmed that obestatin is one of the cleavable targets of DPP-IV.

We hypothesize that MIN6 cells secrete obestatin in some amount which exert incretin effect through paracrine or autocrine signalling. The presence of obestatin in media was observed using ELISA further confirmed that MIN6 cells secrete obestatin (**in previous experiment, table 1**). In fact, paracrine signalling is also reported for GLP-1 suggesting that pancreatic α-cells secrete GLP-1 which acts upon β-cells [21]. It is very important to discuss that in presence of DPP-IV, insulin secretion was decreased profoundly in GLP-1 treated group, as compared to obestatin treated group. Decrease in obestatin concentration was also more pronounced in GLP-1 treated group. These data suggest that DPP-IV cleaved endogenously secreted obestatin and externally provided GLP-1 both in competitive manner, so some amount of GLP-1 rescued from cleavage and contributed for incretin effect. It is also possible that obestatin was secreted in lesser amount in this group, due to presence of GLP-1in extracellular environment, as observed in experiment 1. It is also evident from decrease in obestatin concentration in this group.While, obestatin stimulated insulin secretion more profoundly than GLP-1 because DPP-IV cleaved less amount of obestatin due to less affinity with this substrate. Decrease in insulin and obestatin concentrations in DPP-IV treated group confirmed that obestatin is one of the cleavable targets of DPP-IV, even if having lesser affinity for DPP-IV, as compared to GLP-1.In response to administration of both obestatin and DPP-IV, lesser decrease was found in the concentrations of insulin and obestatin, as compared to GLP-1 and DPP-IV treated group. To understand this outcome we explored three possibilities: (i) obestatin is less preferential target of DPP-IV than GLP-1 (ii) exogenously supplied and endogenously secreted obestatin provided a large amount of substrate to be cleaved by DPP-IV, thus more functional amount of obestatin remained there to exert incretin effect (iii) due to presence of externally available obestatin, initially endogenous secretion of obestatin was diminished (as observed in experiment 1), but simultaneously cleavage of obestatin by DPP-IV, endogenous secretion of obestatin was again taken place, which efficiently participated for incretin effect (it is possible as the study duration was 48 hrs).We have conducted some experiments to understand this outcome, where synthetic substrate(Gly-pro-p-nitroanilide) of DPP-IV and obestatin were used together in various concentrations to look for the inhibition in cleavage of synthetic target due to presence of obestatin. In this study, we found comparably less inhibition in cleavage of synthetic substrate by obestatin, if considered equal amount of both synthetic substrate and obestatin(data not shown). These data supported that GLP-1 is more preferential target of DPP-IV, as compared to obestatin, but still obestatin is also cleavable target of DPP-IV. It seems plausible due the fact that DPP-IV cleaves off dipeptide from N-terminal of peptides if alanine or proline is on position 2, but in case of obestatin it cleaved on amidated C-terminal, where alanine was there on position 2. Therefore, it seems quite possible that GLP-1 is more preferential target of DPP-IV cleavage than obestatin [5, 6]. Mass spectrometric analysis of obestatin incubated with DPP-IV confirmed the cleavage and release of dipeptide (alanine- leucine) from C-terminal of obestatin (**Fig. 1**).

To address relative affinity of GLP-1 and obestatin with DPP-IV and GLP-1R, *in silico* molecular docking studies were also conducted. Data from these studies revealed that obestatin has strong binding potential with DPP-IV and GLP-1 receptor, but this potential was still lesser than the GLP-1 (**Fig 2 and 3**). It seems that gluco-regulation through obestatin is a parallel mechanism to support incretin effect through GLP-1 and mainly dependent on the extracellular availability of GLP-1, glucose and DPP-IV. We conclude that obestatin has functional and structural similarities with GLP-1, but it is more resistant cleavage target of DPP-IV. Development of augmentation therapy for this parallel pathway of glucose regulation or designing obestatin biosimilars may open new arena of diabetes therapy. Further *in vivo* studies are recommended to understand the role of obestatin in physiological context. To confirm whether obestatin binds with GLP-1receptor, study on GLP-1R knockout and wild type cell lines are strongly recommended.

V. Conclusions:

We conclude that obestatin has functional and structural similarities with GLP-1, but it is more resistant cleavage target of DPP-IV. Obestatin has potential to bind with both GLP-1 receptor and DPP-IV enzyme. We hypothesize that development of obestatin based incretin therapies may open new avenues for efficient ant diabetic drug development. Further *in vivo* studies are recommended to understand the role of obestatin in physiological context. To confirm whether obestatin binds with GLP-1receptor, study on GLP-1R knock out and wild type cell lines are strongly recommended.

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