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Enterostatin alters protein trafficking to inhibit insulin secretion in Beta-TC6 cells

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Abstract

Enterostatin is a peptide that regulates dietary fat intake in rodents and inhibits insulin secretion from pancreatic beta cells. Microarray studies of the genomic response of both a human hepatoma cell line (HepG2 cells) and a mouse hypothalamic cell line (GT1-7 cells) to enterostatin suggested that it might regulate protein trafficking. Using semi quantitative real-time PCR and western blot analysis, we confirmed that enterostatin upregulated Scamp2 and down regulated Dynamin2 in these cell lines. The receptor for enterostatin is the F₁-ATPase beta subunit. We transfected HepG2 cells with either a Green Fluorescent protein (GFP) tagged F₁-ATPase beta subunit or a Red Fluorescent protein (RFP) tagged F₁-ATPase alpha subunit to study the effects of enterostatin on translocation of its own receptor protein. Enterostatin induced movement of GFP- beta subunit to the cell periphery area but did not have any effect on the localization of RFP-alpha subunit protein in HepG2. As Scamp2 is involved in glucose uptake in mouse Beta-TC6 insulinoma cells we tested enterostatin's effect in Beta-TC6 cells. Glucose stimulated insulin release was inhibited by enterostatin as reported previously. Using siRNA to Scamp2 did not change glucose stimulated insulin release but siRNA to Dynamin2 and dominant negative Dynamin2 (Dyn K44A) inhibited glucose stimulated insulin release and abolished the response to enterostatin. This suggests enterostatin inhibits glucose stimulated insulin release in pancreatic beta cells through down regulation of Dynamin2. This study also suggests that enterostatin might have a more generalized effect on protein trafficking in various cells.

Keywords

Enterostatin; Dynamin 2; Scamp2; Insulin secretion; Protein trafficking

1. Introduction

Enterostatin is pentapeptide, produced in the exocrine pancreas, stomach and several brain regions [17,27,31], known to induce satiation in rodents and selectively inhibit fat intake [5, 11,18,19]. In addition to its effect in feeding behavior, enterostatin also has multiple other effects. We reported that enterostatin inhibits angiogenesis in both Human Umbilical Vein Endothelial Cells (HUVEC) and fat cells [22] and promotes Myocellular fatty acid oxidation through its stimulation of the AMPK signaling pathway [22]. Enterostatin also inhibits

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forskolin induced insulin secretion in isolated pancreatic islets [20], and it has been reported to enhance memory and inhibit analgesia induced by the μ -opioid agonist morphine [28]. Recently it was reported the oral administration of enterostatin reduces blood cholesterol by inhibiting VLDL and LDL [30]. We and others reported that the F₁-ATPase beta subunit protein is localized on plasma membranes of various tissue and cell lines [14,21] where it may act as a receptor for enterostatin [21]. While we have recently reported that enterostatin can activate MAPKinase ERK and cyclic AMP signaling in neuronal cells [23], there is little information on the mechanisms through which many of the divergent responses to enterostatin are achieved. Using a genome microarray analysis of the response to enterostatin in GT1-7 neuronal and HepG2 liver cells we identified sets of genes involved in angiogenesis and protein trafficking that were responsive to enterostatin. This anti-angiogenic effect of enterostatin that was predicted from the microarray was confirmed using specific angiogenesis assays [22]. The aim of the experiments described in this manuscript was to confirm the effect of enterostatin on protein trafficking that was suggested in the same microarray analysis and investigate the role of 2 of the genes on the enterostatin regulation on insulin secretion. Scamp2 and Dynamin2 were among the protein trafficking genes that responded to enterostatin. Since Scamp2 is known to enhance glucose uptake and Dynamin2 promotes insulin secretion [10], we examined whether enterostatin affected protein trafficking and if the effect of enterostatin on insulin secretion in pancreatic beta TC6 cells is mediated through either Scamp2 or Dynamin2. We also examined the effect of enterostatin on the translocation of its own receptor and investigated if internalization of the receptor was required for the signaling response to enterostatin.

2. Materials and Methods

2.1. Cell culture and Transfection

GT1-7 cells were a gift from Dr. Pamela Mellon (University of California, San Diego). Both HepG2 and Beta-TC6 cells were obtained from American Tissue Culture Collection (Manassas, VA). Cells were grown and maintained in Dulbecco's modified eagle's medium (Hyclone, Logan, UT) containing 10% fetal bovine serum (Hyclone), penicillin (100 unit/ml) and streptomycin (100 μ g/ml). Cells under passages 20 and 60–80% confluent were used in all assays. For real time imaging stimulus of the movement of F₁-ATPase beta subunit or alpha subunit proteins, HepG2 cells were cultured on the Lab-Tek II chambered cover glass (Nalge Nunc International, Rochester, NY) at 50–70% confluency. Small interfering RNA (siRNA) for Dynamin2 and Scamp2 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and siRNA transfection procedures were followed according to the manufacture's instruction. Cells were washed with 2 ml of transfection media (Santa Cruz Biotechnology Inc.) before transfection. Then, 6 μ l (60 pmol) of siRNA (Dynamin2, Scamp2 or control scrambled siRNA) in transfection media was mixed with 6 μ l of transfection reagent (Santa Cruz Biotechnology Inc.) for 45 min at room temperature. After 5 hours transfection 2X normal media (supplemented with 2 times of FBS concentration) was added. Fresh normal growth media was added to the cells after 18 hour transfection. Cells were then assayed with or without enterostatin post 72 hours transfection. Dominant negative Dynamin2 (K44A) was a kind gift from Dr. Horazdovsky (Mayo Clinic). Beta TC-6 cells were transfected with 2 μ g of DN Dny2 (K44A) plasmid and 5 μ l of Lipofectamin2000 (Invitrogen) according to the manufacturer's instructions and these cells used 48 hours later for studies of insulin secretion and enterostatin signaling pathways.

2.2. Plasmid Construction

Rat F₁-ATPase β -subunit clone (1,254bp) was generated by PCR from an amygdala cDNA library [21] using F₁-ATPase β -subunit forward primer (5' GAGAGGAGCTCCACTATTGCTATGGATGGC3', Sac I recognition site underlined) and F₁-ATPase β -subunit reverse primer (5'GAGAGAAGCTTCACGACCCATGCTC3', Hind III

recognition site underlined). The PCR product was cloned into the pEGFP-C1 vector (Clontech, Palo Alto, CA) between the Sac I and Hind III sites (Figure 1A) and then transformed into DH5 α cells. Transformants were screened and positive clones containing plasmid with the correct orientation of the insert were cultured. The F₁-ATPase α -subunit clone (1,662bp) was generated by PCR from an amygdala cDNA library using F₁-ATPase α -subunit forward primer (5'GAGGGAGCTCAGCTGCAAGGATGCTGTCC3', Sac I recognition site underlined) and F₁-ATPase α -subunit reverse primer (5' GAGAGAAGCTTTTACCGTTCAAACCCAGC3', Hind III recognition site underlined). The PCR product was cloned into the pDsRed2-C1 vector (Clontech) between the Sac I and Hind III sites (Figure 1B) and then transformed into DH5 α cells. Transformants were screened and positive clones containing plasmid with the correct orientation of the insert were cultured with antibiotics. For transfection, 0.8 μ g of either the pATPase β subunit-GFP or pATPase α subunit-RFP plasmid DNA and 2.4 μ l of Fugene 6 (Roche, Indianapolis, IN) were incubated in 100 μ l of Opti-MEM (Invitrogen) for 30 min at room temperature in 1 ml total volume of media for 16–24 h.

2.3. Microarray and Semi quantitative RT-PCR

For Microarray, GT1-7 and HepG2 cells were grown in 75cm² flask; cells were then incubated in the absence of or with various doses of enterostatin (0.01, 0.1 and 1.0 μ M) for 1 hour. Cells were harvested, RNA was extracted using TriReagent (Molecular Research, Cincinnati, OH), treated with Turbo DNase (Ambion, Austin, TX) and cleaned by RNeasy columns (Qiagen, Valencia, CA). RNA quality was visually assessed using agarose gel electrophoresis and quantified by UV spectrophotometric analysis (A₂₆₀ and A₂₈₀ nm). All RNA had A₂₆₀/A₂₈₀ ratios greater than 1.75 and less than 2.10. RNA integrity was checked using on RNA 6000 nano lab chip kit (Agilent Technologies, Foster City, CA, USA). Details of the microarray analysis were reported previously [22]. Briefly, the high density microarrays used in this study were generated by the Genomics Core Microarray Facility at the Pennington Biomedical Research Center using a Gene Machines OmniGrid Microarrayer (San Carlos, CA, USA) to spot 70-mer oligonucleotides (mouse library versions 1.0 and 2.0, Operon Biotechnologies, Inc., Huntsville, AL, USA) onto poly-lysine-coated glass microscope slides. The mouse libraries used for printing slides represent over 19,000 well-characterized genes; information concerning gene specifics can be located on the Operon website (<http://omad.operon.com/mouse/index.php> and <http://omad.operon.com/mouse2/index.php>). Equal amounts (6 μ g) of total RNA from each of the enterostatin doses and the control untreated cells was subjected to reverse transcription with oligo(dT), labeled with Cy3 and Cy5 dyes using the Micromax TSA™ Labeling and Detection Kit protocol (Perkin Elmer Life Sciences, Inc., Boston, MA, USA) and hybridized to in-house spotted slides. The TSA™ labeling method uses a tyramide signal amplification process, and is highly sensitive, allowing for the use of very small amounts (as little as 2 μ g) of total RNA with consistent and reproducible signal amplification across arrays. cDNA from enterostatin treated groups (10, 100, 1000 nM enterostatin) were labeled with Cy5 and hybridized against control cDNA labeled with Cy 3. Control-Cy5 labeled cDNA was also compared to control Cy3 cDNA. Slides were scanned using a GSI Lumonics ScanArray 5000 laser scanner (Perkin Elmer Life Sciences, Inc., Boston, MA, USA) at a relatively low and a relatively high laser intensity and expression data was analyzed using the QuantArray V3.0 software package (Perkin Elmer Life Sciences, Inc., Boston, MA, USA). The data were then normalized using a subarray-by-subarray LOWESS (Locally Weighted Regression and Scatterplot Smoother) statistical algorithm developed in house at the Pennington Biomedical Research Center. Only genes with 2 fold up or down regulation that showed a dose response to enterostatin were included in the Panther Pathway analysis (Applied Biosystems).

DNA free RNA was reverse transcribed using MMLV reverse transcriptase (Promega, Madison, WI) and the resulting cDNA analyzed by semi-quantitative PCR using gene specific

primer sets. The PCR product was run on 3% agarose gel and the band intensities were measured by Quantity One (BioRad, Hercules, CA). The following primer sequences were used for mouse Dynamin2; forward 5'-GCCTCCCCTGATTCCTATGC-3' and reverse 5'-TCCGTGCTGGCCGAGAT-3', human Dynamin2; forward 5'-GCCCCCCTGATTCCTGTTTC-3' and reverse 5'-TCCGAACTGGCCGAGAT-3', mouse Scamp2; forward 5'-TGACTACCAGCGGATTTGCA-3' and reverse 5'-ACGCAAGCAGGTTTAGAAA-3' and human Scamp2; forward 5'-CGACTACCAGCGGATATGCA-3' and reverse 5'-AGGCAAGCAGGTTTCAGAAA-3'. Mouse Cyclophilin B as an internal control; forward GGCTACAAAAACAGCAAGTTCCAT-3' and reverse 5'-GCTCTCCACCTTCCGTAC-3' or human Cyclophilin B; forward 5'-GGAGATGGCACAGGAGGAAA-3' and reverse 5'-CGTAGTGCTTCAGTTTGAAGTTCTCA-3'. To verify the inhibition of gene expression by siRNA we purchased appropriate primers for Scamp2 (sc-41293-PR) and for Dynamin2 (sc-35237-PR) from Santa Cruz biotechnology.

2.4. Plasma membrane and mitochondrial membrane isolation from HepG2 cells

To prepare plasma membrane fractions, HepG2 cells were washed three times with 200 μ l of extraction buffer on ice (10mM HEPES, pH 7.5, containing 200mM mannitol, 70mM sucrose, and 1mM EGTA) before homogenization. The washed tissue was homogenized in 10 volumes of ice-cold homogenization buffer (extraction buffer containing Mini-complete protease cocktail to final concentration of 0.5mg/ml, [Roche, Indianapolis, IN]) using a Potter-Elvehjem Teflon-glass homogenizer. The homogenates were centrifuged at 600g for 5min at 4°C, the supernatants removed and then centrifuged at 16,000g for 20min at 4°C to yield the mitochondrial fraction. The 16,000g supernatants containing plasma membrane and cytosolic components were centrifuged at 100,000g for 1hr at 4°C to sediment the plasma membrane fractions. The fractions containing the plasma membrane and the mitochondrial membrane were checked for purity using the marker enzymes alkaline phosphatase and cytochrome c oxidase respectively (Sigma Chemical Co., St Louis, MO) as described previously [21].

2.5. Western Blots

Scamp2 and Dynamin2 antibodies were obtained from Abcam (Cambridge, MA). Polyclonal antibodies to F₁-ATPase beta and alpha subunits were generated as described previously [21] and are available through Echelon Bioscience (Salt Lake City, Utah). The pERK antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and pPKA α antibody from BD Bioscience (San Jose, CA). For immunoblotting, we used protocols described previously [22,23]; 50 μ g of total protein or membrane fraction [21] for each assay were separated on 10% SDS-PAGE and, proteins were transferred to PVDF membrane for 3hours at 80V. The blots were incubated with appropriate primary antibodies overnight at 4°C. Membrane was then incubated with the secondary antibody before exposure to the X-ray film (X-Omat Kodak New Haven, CT).

2.6. Enterostatin signaling

Wild type or transfected Beta TC-6 cells maintained in Dulbecco's modified medium were incubated with enterostatin for 15 minutes, the cells harvested, washed twice with cold phosphate-buffered saline, 200 μ l of ice-cold whole cell lysis buffer (50mM KCl, 1% NP-40, 25mM HEPES-pH7.8, 10 μ g/ml Leupeptin, 20 μ g/ml Aprotinin, 125 μ M DTT, 1mM PMSF and 1mM Orthovanadate) added and the cells recovered using a spatula. Cells were sonicated and centrifuged at 4°C and 14,000 rpm for 15min to obtain total protein. This protein was used for Western Blot analysis of the phosphorylation of ERK and PKA α as previously described [23].

2.7 Insulin secretion

Wild type or transfected Beta TC-6 cells were incubated in Dulbecco's modified eagle's media containing glucose (25mM) as described above. Enterostatin or vehicle was added and insulin secreted into the media assayed after 1 hour using a rat Insulin RIA (Linco Research St. Charles, MO). Radioactivity was counted using a Wallac 1470 Wizard gamma counter (Turku, Finland). Cells were collected for protein quantification using BCA kit (Pierce, Rockford, IL).

2.8 Immunohistochemistry

Cells were cultured on Poly-L-Lysine coated 18mm-coverslip. Acid washed cover slips were incubated with Poly L-lysine (1:10 dilution, Sigma) for 20 min at room temperature with shaking and coated cover slips were washed twice with PBS and dried under the hood. Cells cultured on the cover slips were incubated with or without enterostatin for 1 hour at 37°C with 5% CO₂. After incubation, cells were washed three times with PBS, fixed with 4% paraformaldehyde (Sigma) in PBS for 20 min at room temperature (RT) and then received 3 further washes with PBS. Cells were blocked in 10% goat serum (Sigma) in PBS for 1 hr at RT, incubated for 3h at 37°C with antibodies to Dynamin2 or Scamp2 in 5% goat serum, washed 3 times with PBS then incubated for 1 hr at 37°C with the secondary antibody (Alexa Fluor 594, Invitrogen, Carlsbad, CA) in 1:500 ratios in 5% goat serum. Cells were stained with DAPI (4', 6-diamidino-2-phenylindole, 100ng/ml, Sigma) for 5 min at RT. Cover slips were mounted on each slide using Vecta Shield Fluorescence Mounting Media H-1400 (Vector labs, Burlingame, CA). Controls included preneutralization of antibody with excess primary antigens and omission of primary antibodies. To capture images, we either used a Zeiss Axioplan 2 imaging system, (Zeiss, Thornwood, NY), or a Leica DM IL with Leica EL6000 fluorescent light source (Bannockburn, IL). In order to image the live cells in real time we used a confocal microscope (Zeiss NSM 510 META) with an inverted camera.

3. Results

3.1. Microarray data suggests genes related to protein trafficking were altered by enterostatin in both GT1-7 and HepG2 cells

Only genes that showed a > 2-fold up or down-regulated in response to enterostatin and showed a dose related response to enterostatin (0.01 to 1.0μM) were used in the pathway analysis. These data (Table 1) showed 51 genes related to protein trafficking in HepG2 and 77 genes in GT1-7 that were regulated by enterostatin. Among the genes identified there was increased expression of the secretory protein SCAMP2 and decreased expression of Dynamin2 and nuclear importing protein (karyopherin [importin] alpha 2) in cells treated with enterostatin for 1 hour.

3.2. Enterostatin effects on Scamp2 and Dynamin2 expression in HepG2 and Beta-TC6 cells

Since Scamp2 and Dynamin2 were among those genes altered by enterostatin that were identified in the microarray analysis, we confirmed the response by using quantitative PCR and Western blot analysis. Incubation of HepG2 cells with enterostatin for 1 hour showed a dose-related increase in Scamp2 gene and protein expression and a dose related decrease in Dynamin2 gene and protein expression (Fig 2A and B). Immunohistochemical analysis of Beta TC6 cells incubated in the presence or absence of enterostatin also showed an increase in Scamp2 and decrease in Dynamin2 levels in cells incubated with enterostatin (Fig 2C).

3.3. Enterostatin alters F₁-ATPase beta subunit localization but not the alpha subunit in HepG2 cells

HepG2 cells transfected with either alpha subunit-RFP or beta subunit-GFP were incubated with either 0.5 or 2 μM enterostatin. After 15, 30, or 60 min of incubation with enterostatin,

the cover slip was removed from the well and placed on a glass slide. Protein translocation in HepG2 cells was then observed using confocal microscopy. Enterostatin had no effect on the distribution of expressed F₁-ATPase alpha subunit-RFP in HepG2 cells, the protein remained diffusely spread throughout the cells (Fig 3 cells C and D). In contrast, the F₁-ATPase beta subunit protein was more localized towards the center of cells initially (Fig 3A, cells A and B) and was translocated towards its periphery after incubation with enterostatin. Western blot analysis also showed the presence of F₁-ATPase beta subunit protein in both mitochondrial and plasma membrane fractions but not in the cytosolic fraction. The plasma membrane fraction was free of cytochrome oxidase activity ruling out possible contamination with mitochondria. Cells incubated with enterostatin had increased levels of F₁-ATPase beta subunit protein (50kDa) in the plasma membrane fraction (Figure 4). The band at 37kDa which cross reacted with the antibody was only present in the plasma membrane fractions and it too was increased in level by enterostatin. We do not know the identity of this protein but assume it may be a cleavage product from the parent protein.

3.4. Changes in Scamp2 and Dynamin2 expression levels in Beta-TC6 cells are related to the Enterostatin effect on Insulin secretion

As Scamp 2 and Dynamin 2 are involved in both endo- and exocytosis and Dynamin 2 has been related to insulin secretion [3,7,10,13,24] we investigated the possibility that the enterostatin inhibition of glucose stimulated insulin secretion might be mediated through its effects on Scamp2 or Dynamin 2. siRNA to Dynamin2 induced a major knockdown of Dynamin2 levels in Beta-TC6 cells whereas the siRNA to Scamp2 was somewhat less effective in knocking down Scamp2 (Figure 5A). Knockdown of Dynamin2 had little effect on basal insulin secretion (not shown) but significantly inhibited glucose induced insulin secretion while siRNA to Scamp2 did not change insulin secretion (Figure 5B). Enterostatin inhibited glucose induced insulin secretion. This response was unaffected by Scamp2 knockdown but was abolished when Dynamin2 expression was suppressed. We further used a dominant negative Dynamin2 (Dyn K44A) construct to confirm the effect of Dynamin2 on insulin secretion and the response to enterostatin. The dominant negative Dynamin2 reduced glucose induced insulin secretion by 50% and abolished the response to enterostatin (Figure 5C).

3.5. Is Dynamin2 important for Enterostatin's effects on signaling pathways?

We had previously shown that enterostatin stimulates ERK phosphorylation and the cAMP signaling pathway [23]. The mechanism through which binding to F₁-ATPase beta subunit protein on the plasma membrane initiates these signaling responses is unclear. It is possible that signaling is dependent upon an endocytic event to encapsulate a signaling endosome [6, 32]. Since Dynamin 2 is required for internalization of signaling endosome [25] we investigated the signaling response to enterostatin in cells transfected with the dominant negative Dynamin 2 construct. However, enterostatin activated PKA_{R11β} and increased pERK level in cells transfected with the DN Dynamin2 (Figure 6) suggesting that enterostatin did not need internalization of its receptor for signaling.

4. Discussion

The F₁-ATPase beta subunit protein is an integral part of ATP synthase within the mitochondria. However, a number of reports, including data from our laboratory, have shown that both this beta and the alpha subunit proteins of F₁-ATP synthase are present in plasma membranes of a variety of cells where they may act as receptors or transporters for Apolipoprotein A and angiostatin. [2,14,15]. We and others have shown that the F₁-ATPase beta subunit also binds enterostatin [1,21] and that through binding to this protein enterostatin affects both cyclic AMP and MAPKinase signaling pathways [23]. The beta subunit protein is synthesized on the endoplasmic reticulum and must be trafficked to both the mitochondrial

and plasma membrane locations. The data we present in this manuscript suggests that enterostatin itself may act as a signal to increase the trafficking of the protein to the plasma membrane location. Using both Western blots and confocal imaging of cells transfected with a beta subunit-green fluorescent protein construct, we provide evidence that enterostatin increases the level of beta subunit protein in the plasma membrane fraction and stimulates trafficking of the protein towards the periphery of cells. In contrast, enterostatin had no effect on the distribution of an alpha subunit-red fluorescent protein construct suggesting it was selective in its effects. These data confirm evidence recently reported data by Lindquist and colleagues [12] which also suggested that both enterostatin and certain fatty acids upregulated the level of the beta subunit in plasma membrane of INS-1 insulin secreting cells. We do not know the consequences of this translocation of F₁-ATPase beta subunit in HepG2 cells to the plasma membranes. According to Martinez and colleagues [14], plasma membrane F₁-ATPase beta subunit also serves as an Apolipoprotein AI receptor and is involved in cholesterol transport. This would be consistent with the report that enterostatin reduces plasma cholesterol levels in vivo [29] and would suggest that increased localization of the beta subunit protein to the plasma membrane might be important for this response.

Gene array analysis of the response to enterostatin of two cell lines identified a number of genes that were linked to membrane protein trafficking. We chose to focus on Dynamin 2 and Scamp2 since these are known to be integrally involved in endocytic and exocytic mechanisms [3,7,9,10]. Dynamin 2, a ubiquitously expressed protein is a microtubule associated GTPase crucial in the early steps of endocytosis [3]. Scamp 2 is associated with the pool of Glut 4 transporters in the cell and appears to be important for the recycling of receptors to the intracellular pools [7,9,24]. We confirmed the microarray data that enterostatin increased expression of Scamp2 and reduced expression of Dynamin2 by RTPCR and further showed that these genomic changes were reflected in changes in the level of the respective proteins in the cells. Further, as both gene products are known to affect glucose stimulated insulin secretion [10], we questioned the possibility of their involvement in the enterostatin inhibition of insulin secretion. We showed that both down regulation of Dynamin2 gene expression and a dominant negative Dynamin 2 inhibited glucose stimulated insulin secretion in agreement with a previous report [10]. Knockdown of Dynamin 2 also blocked any inhibitory effect of enterostatin on insulin secretion. This is consistent with the hypothesis that the enterostatin inhibition of insulin secretion is mediated by its effects in down regulating dynamin2 gene expression. However, this is unlikely to fully explain the effects of enterostatin on insulin secretion. Enterostatin acutely inhibits both first and second phase of insulin secretion from isolated islets and perfused pancreas [4,20,26] as well as having long term effects in vivo [16]. The acute effects of enterostatin on first phase insulin expression cannot be explained by inhibition of Dynamin 2 gene expression and reduction in Dynamin 2 protein levels within the cell. However, enterostatin had a major impact on Dynamin 2 mRNA and protein levels within one hour suggesting that this very rapid effect might contribute towards the inhibition of 2nd phase insulin secretion and certainly towards the chronic effects of enterostatin on insulin secretion. Although Scamp2 has been associated with the intracellular localization of Glut4 glucose transporters [7,8,24], we did not observe any effects of knockdown of Scamp 2 on either basal, glucose stimulated or enterostatin inhibition of insulin secretion. This suggests that there was insufficient knockdown of gene expression to alter the uptake of glucose into the cell.

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A. F₁-ATPase β subunit-GFP construct

B. F₁-ATPase α subunit-RFP construct

Figure 1.

F₁-ATPase beta subunit-Green Fluorescent Protein (GFP) construct (A) and F₁-ATPase alpha subunit-Red Fluorescent Protein (RFP) construct (B).

Figure 2.

Effect of enterostatin on gene expression (A) and protein expression (B) of Dynamin2 and Scamp2 in HepG2 cells. Section C shows immunohistochemical images of Scamp2 and Dynamin2 together with nuclei (DAPI staining) of HepG2 cells incubated in presence or absence of enterostatin. All incubations were for one hour. The values shown in panel A are Means $\pm$ SEM for 8 observations in each group.

Figure 3.
Confocal microscopic images of F₁-ATPase beta subunit protein (Green) or alpha subunit protein (Red) in HepG2 cells incubated with (cells A through D; bottom images) or without enterostatin (1 μM) (cells A through D; top images) for one hour.

Figure 4.

Western blot of F₁-ATPase beta subunit in subcellular fractions of HepG2 cells incubated in the presence or absence of enterostatin for 1 hour. 20μg protein from plasma membrane (PM), light mitochondrial fraction (MT) or cytosolic fraction (CYT) was loaded onto the gel. Marker assays showed the MT and PM fractions were free of cross contamination and neither MT or PM marker activity was evident in the cytosolic fraction..

Figure 5.

Effects of Dynamin 2 and Scamp 2 on insulin secretion from Beta-TC6 cells. Panel A shows knockdown of Dynamin 2 and Scamp2 by their respective siRNAs (D and S) compared to a control (C) scrambled siRNA. Panel B shows effects of siRNA to Scamp2 and Dynamin 2 on glucose stimulated insulin secretion and on the inhibitory response to enterostatin. Panel C shows that a transfected Dominant Negative Dyn2 (K44A) construct (DN) inhibits insulin secretion and blocks any further inhibitory effect of enterostatin on insulin secretion. Values represent Means $\pm$ SEMs for a minimum of 4 observations/group. *** $p < 0.001$ compared to controls or groups as shown.

Figure 6.

The lack of effect of a dominant negative Dynamin 2 [DN Dyn2 (K44A)] on the enterostatin stimulation of phospho-PKA_{RIIβ} and pERK in Beta TC-6 cells. Wild type (DN -) or dominant negative Dynamin 2 transfected cells (DN +) were incubated 48 hours after transfection with enterostatin (0.01 or 1.0 μM) for 15 minutes.

Table 1
Genes involved in Protein Trafficking in response to Enterostatin

Gene Name	Symbol	GT1-7	HepG2
ARP1 actin-related protein 1 homolog A (yeast)	Actr1a	nd	down
annexin A7	Anxa7	up	nd
adaptor protein complex AP-1, beta 1 subunit	Ap1b1	up	nd
adaptor protein complex AP-1, gamma 1 subunit	Ap1g1	down	down
adaptor protein complex AP-1, gamma 2 subunit	Ap1g2	down	down
adaptor protein complex AP-1, sigma 1	Ap1s1	up	nd
adaptor-related protein complex 2, beta 1 subunit	Ap2b1	up	nd
adaptor-related protein complex 3, sigma 1 subunit	Ap3s1	up	nd
adaptor-related protein complex AP-4, mu 1	Ap4m1	down	nd
ADP-ribosylation factor 1	Arf1	down	up
ADP-ribosylation factor 5	Arf5	down	nd
ADP-ribosylation factor 6	Arf6	down	up
Rho guanine nucleotide exchange factor (GEF) 1	Arhgef1	down	nd
ADP-ribosylation factor-like 2	Arl2	down	up
ADP-ribosylation factor-like 6	Arl6	nd	up
blocked early in transport 1 homolog (S. cerevisiae)	Bet1	down	nd
choroideremia	Chm	up	nd
clathrin, light polypeptide (Lca)	Clta	up	down
component of oligomeric golgi complex 2	Cog2	up	nd
coatamer protein complex, subunit beta 1	Copb1	nd	up
coatamer protein complex, subunit epsilon	Cope	nd	up
coatamer protein complex, subunit gamma 2	Copg2	down	nd
chromosome segregation 1-like (S. cerevisiae)	Cse1l	nd	up
casein kinase 1, alpha 1	Csnk1a1	nd	up
casein kinase 1, delta	Csnk1d	up	up
dynactin 1	Dctn1	nd	down
dynein, cytoplasmic, heavy chain 1	Dnchc1	nd	down
dynein, cytoplasmic, intermediate chain 1	Dncic1	nd	up
dynamitin 1	Dnm1	down	nd
dynamitin 2	Dnm2	down	down
epimorphin (SYNTAXIN 2)	Epim	up	nd
endoplasmic reticulum protein 29	Erp29	down	nd
exocyst complex component 7	Exoc7	up	nd
GTPase activating RANGAP domain-like 1	Garnl1	nd	down
guanosine diphosphate (GDP) dissociation inhibitor 2	Gdi2	up	nd
guanine nucleotide binding protein-like 2 (nucleolar)	Gnl2	up	up
golgi autoantigen, golgin subfamily a, 3	Golga3	down	nd
golgi autoantigen, golgin subfamily a, 4	Golga4	up	nd
GRP1 (general receptor for phosphoinositides 1)-associated scaffold protein	Grasp	down	nd
general transcription factor II A, 1-like factor	Gtf2a1	nd	down

Gene Name	Symbol	GT1-7	HepG2
general transcription factor II A, 1-like factor	Gtf2a1lf	down	down
Huntington disease gene homolog	Hdh	up	nd
kinesin family member 20A	Kif20a	nd	up
kinesin family member 3B	Kif3b	up	nd
kinesin family member 3C	Kif3c	up	nd
kinesin family member 5C	Kif5c	nd	down
karyopherin (importin) alpha 2	Kpna2	nd	down
low density lipoprotein receptor-related protein 1	Lrp1	down	down
mitsugumin 29	Mg29	up	up
N-ethylmaleimide sensitive fusion protein attachment protein alpha	Napa	up	nd
natural killer tumor recognition sequence	Nktr	down	nd
neutral sphingomyelinase (N-SMase) activation associated factor	Nsmaf	up	nd
nucleoporin 62	Nup62	up	up
peroxisomal biogenesis factor 3	Pex3	up	nd
peroxisome biogenesis factor 5	Pex5	up	nd
peroxisomal biogenesis factor 6	Pex6	nd	down
phosphatidylinositol transfer protein, membrane-associated 2	Pitpm2	up	up
peptidylprolyl isomerase A	Ppia	nd	up
peptidylprolyl isomerase C	Ppic	up	nd
peptidylprolyl isomerase E (cyclophilin E)	Ppie	down	nd
pleckstrin homology, Sec7 and coiled-coil domains 3	Pscd3	up	nd
PX domain containing serine/threonine kinase	Pxk	up	nd
RAB10, member RAS oncogene family	Rab10	down	down
RAB11a, member RAS oncogene family	Rab11a	nd	down
RAB11B, member RAS oncogene family	Rab11b	down	down
RAB14, member RAS oncogene family	Rab14	down	nd
RAB18, member RAS oncogene family	Rab18	up	nd
RAB37, member of RAS oncogene family	Rab37	down	nd
RAB3C, member RAS oncogene family	Rab3c	nd	down
RAB3D, member RAS oncogene family	Rab3d	up	up
1 RAB4A, member RAS oncogene family	Rab4a	up	up
RAB5A, member RAS oncogene family	Rab5a	down	nd
RAB5B, member RAS oncogene family	Rab5b	down	nd
RAB6, member RAS oncogene family	Rab6	nd	up
guanine nucleotide exchange factor (GEF) 1	Rabgef1	down	nd
RAN binding protein 1	Ranbp1	down	nd
SAR1a gene homolog 1 (S. cerevisiae)	Sara1	down	nd
secretory carrier membrane protein 2	Scamp2	nc	up
secretory carrier membrane protein 3	Scamp3	nd	up
secretory carrier membrane protein 5	Scamp5	up	up
syndecan binding protein (syntenin) 2	Sdcbp2	up	nd
SH3 domain protein 1B (INTERSECTIN 2)	Sh3d1B	down	nd

Gene Name	Symbol	GT1-7	HepG2
synaptosomal-associated protein 23	Snap23	down	nd
sorting nexin 1	Snx1	up	nd
sorting nexin 2	Snx2	down	down
sorting nexin 5	Snx5	down	nd
sorting nexin 9	Snx9	down	nd
syntaxin 5A	Stx5a	down	nd
syntaxin 6	Stx6	down	nd
syntaxin 8	Stx8	up	nd
synaptotagmin 10	Syt10	down	up
synaptotagmin 8	Syt8	nd	down
trafficking protein particle complex 4	Trappc4	nd	up
tubulin, alpha 4	Tuba4	nd	up
tubulin, beta 3	Tubb3	nd	up
vesicle-associated membrane protein, associated protein A (Vapa)	Vapa	up	nd
vesicle-associated membrane protein 4	Vamp4	up	nd
vesicle-associated membrane protein 5	Vamp5	up	up
vesicle-associated membrane protein 8	Vamp8	down	nd
vesicle-associated membrane protein, associated protein B and C	Vapb	down	nd
vesicle docking protein (Interim)	Vdp	nd	up
vacuolar protein sorting 4b (yeast)	Vps4b	up	nd
visinin-like 1	Vsnl1	nd	up
vesicle transport through interaction with t-SNAREs 1B homolog	Vtilb	nd	up
exportin 7	Xpo7	nd	up

ND not detected; up down reflect upregulation or downregulation by enterostatin.