

Regulation of Pancreatic Beta Cell Mass and Function by Proghrelin
Derived Peptides

by

Stephanie Nelson

Submitted in partial fulfillment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
August 2010

© Copyright by Stephanie Nelson, 2010

DALHOUSIE UNIVERSITY

Department of Physiology and Biophysics

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled “Regulation of Pancreatic Beta Cell Mass and Function by Proghrelin Dervied Peptides” by Stephanie Nelson in partial fulfillment of the requirements for the degree of Master of Science.

Dated: August 5, 2010

Supervisor: _____

Readers: _____

DALHOUSIE UNIVERSITY

DATE: August 5, 2010

AUTHOR: Stephanie Nelson

TITLE: Regulation of Pancreatic Beta Cell Mass and Function by Proghrelin
Dervied Peptides

DEPARTMENT OR SCHOOL: Department of Physiology and Biophysics

DEGREE: MSc CONVOCATION: October YEAR: 2010

Permission is herewith granted to Dalhousie University to circulate and to have copied for non-commercial purposes, at its discretion, the above title upon the request of individuals or institutions.

Signature of Author

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

The author attests that permission has been obtained for the use of any copyrighted material appearing in the thesis (other than the brief excerpts requiring only proper acknowledgement in scholarly writing), and that all such use is clearly acknowledged.

TABLE OF CONTENTS

| | |
|---|------|
| LIST OF FIGURES | vii |
| ABSTRACT | viii |
| LIST OF ABBREVIATIONS USED | ix |
| ACKNOWLEDGMENTS | xi |
| CHAPTER 1 INTRODUCTION..... | 1 |
| 1.1 GENERAL BACKGROUND | 1 |
| 1.2 STRUCTURE AND PROCESSING OF PROGHRELIN | 3 |
| 1.3 RECEPTORS FOR PROGHRELIN DERIVED PEPTIDES BEYOND GHS R1A..... | 7 |
| 1.3.1 Unacylated Ghrelin is Not a Ligand for GHS R1a | 7 |
| 1.3.2 Evidence for Unknown Receptors for Acylated Ghrelin | 7 |
| 1.3.3 Unacylated Ghrelin Receptor | 9 |
| 1.3.4 Obestatin Receptor | 10 |
| 1.3.5 Further Controversies on Obestatin's Receptor | 11 |
| 1.3.6 Reports of GHS R1a Dimerization | 11 |
| 1.4 RECEPTOR AND SIGNALING PATHWAY IN ACYLATED GHRELIN STIMULATED GH SECRETION..... | 12 |
| 1.5 GHRELIN AND FOOD INTAKE | 13 |
| 1.6 ANIMAL MODELS OF GHRELIN ACTION | 14 |
| 1.6.1 PGDP Axis KO Models | 14 |
| 1.6.2 Additional Approaches to Animal Models of Reduction or Excess of PGDP | 16 |
| 1.7 GHRELIN AND GLUCOSE HOMEOSTASIS | 17 |
| 1.7.1 Affect of PGDP on Insulin Secretion <i>In Vivo</i> | 19 |
| 1.7.2 Pancreatic Ghrelin | 20 |
| 1.7.3 Molecular Regulation of Insulin Secretion | 22 |
| 1.7.4 PGDP Regulation of Insulin Secretion is Dose and Glucose Dependent..... | 26 |
| 1.8 PROGHRELIN DERIVED PEPTIDES MODULATE BETA CELL MASS | 27 |
| 1.8.1 Beta Cell Mass Fluctuates With Insulin Demand | 27 |
| 1.8.2 Beta Cell Mass and Type 2 Diabetes | 28 |

| | | |
|---------------------------------------|---|----|
| 1.8.3 | Akt Signaling is an Important Pathway in the Maintenance of Beta Cell Mass | 29 |
| 1.8.4 | Apoptosis and Beta Cell Mass | 30 |
| 1.8.5 | PGDP Regulate Beta Cell Mass | 32 |
| 1.9 | STATEMENT OF OBJECTIVES | 33 |
| CHAPTER 2 MATERIALS AND METHODS | | 35 |
| 2.1 | CELL CULTURE AND REAGENTS | 35 |
| 2.2 | GHS R1A DETECTION | 36 |
| 2.2.1 | rt-PCR | 36 |
| 2.2.2 | Western Blot | 37 |
| 2.3 | PALMITATE PREPARATION | 38 |
| 2.4 | PROLIFERATION | 38 |
| 2.4.1 | Cell Counts | 38 |
| 2.4.2 | BrdU Assay | 39 |
| 2.5 | APOPTOSIS | 40 |
| 2.5.1 | Hoechst Nuclear Staining | 40 |
| 2.5.2 | Caspase 3 Assay | 41 |
| 2.6 | AKT SIGNALING | 41 |
| 2.7 | INSULIN SECRETION | 42 |
| 2.8 | EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS | 43 |
| CHAPTER 3 RESULTS | | 44 |
| 3.1 | GHS R1A IS PRESENT IN MIN6 CELLS, BUT NOT INS-1 CELLS..... | 44 |
| 3.2 | BOTH ACYLATED GHRELIN AND UNACYLATED GHRELIN INCREASE/ PRESERVE CELL NUMBER IN INS-1 AND MIN6 CELLS | 47 |
| 3.3 | PROGHRELIN DERIVED PEPTIDES INCREASE PROLIFERATION IN INS-1, BUT NOT MIN6 CELLS | 52 |
| 3.4 | PALMITATE INCREASES APOPTOSIS IN INS-1 AND MIN6 CELLS, WHICH IS REDUCED WITH ACYLATED GHRELIN TREATMENT | 56 |
| 3.5 | ACTIVATED CASPASE 3 IS ELEVATED IN SERUM DEPRIVED INS-1 CELLS AND IS NORMALIZED WITH ACYLATED GHRELIN TREATMENT..... | 59 |
| 3.6 | CASPASE 3 IS NOT ELEVATED IN MIN6 CELLS FOLLOWING 48 HOUR SERUM STARVATION | 64 |
| 3.7 | PALMITATE TREATMENT INCREASES APOPTOSIS IN INS-1 CELLS | 67 |

| | | |
|----------------------------|---|----|
| 3.8 | ACYLATED GHRELIN AND UNACYLATED GHRELIN INCREASE PHOSPHORYLATION OF AKT IN INS-1 AND MIN6 CELLS | 67 |
| 3.9 | INSULIN SECRETION IS MODULATED BY PROGHRELIN DERIVED PEPTIDES IN A GLUCOSE DEPENDENT MANNER IN INS-1 CELLS | 72 |
| 3.9.1 | Acylated Ghrelin Inhibits Insulin Secretion | 81 |
| 3.9.2 | Unacylated Ghrelin Inhibits and Stimulates Insulin Secretion | 81 |
| 3.9.3 | Obestatin Inhibits and Stimulates Insulin Secretion..... | 82 |
| CHAPTER 4 DISCUSSION | | 83 |
| 4.1 | FURTHER EVIDENCE FOR UNKNOWN PROGHRELIN DERIVED PEPTIDE RECEPTORS | 83 |
| 4.1.1 | Acylated Ghrelin Decreases Apoptosis in the Presence and Absence of GHS R1a | 84 |
| 4.1.2 | INS-1 Cells Lack GHS R1a and Respond to Acylated Ghrelin Differently than Their Clonal Derivatives | 84 |
| 4.1.3 | Acylated Ghrelin and Unacylated Ghrelin Increase Cell Number in the Presence and Absence of GHS R1a..... | 85 |
| 4.2 | PROGHRELIN DERIVED PEPTIDES REGULATE BETA CELL MASS IN INS-1 AND MIN6 CELLS..... | 86 |
| 4.2.1 | Effects of PGDP on Proliferation | 86 |
| 4.2.2 | Effects of PGDP on Cell Survival | 88 |
| 4.2.3 | Summary of PGDP Effects on Beta Cell Mass in INS-1 Cells...90 | |
| 4.2.4 | Summary of PGDP Effects on Beta Cell Mass in MIN6 Cells...90 | |
| 4.2.5 | Lipotoxicity and Apoptosis | 91 |
| 4.2.6 | INS-1 and MIN6 Differ in Their Sensitivity to Metabolic Stressors | 92 |
| 4.2.7 | Activation of Akt Signaling in INS-1 and MIN6 Cells | 92 |
| 4.3 | PROGHRELIN DERIVED PEPTIDES MODULATE INSULIN SECRETION IN INS-1 CELLS..... | 93 |
| 4.3.1 | Ghrelin Regulation of Insulin Secretion | 93 |
| 4.3.2 | Obestatin Regulation of Insulin Secretion | 96 |
| 4.4 | CONCLUSION | 97 |
| REFERENCES | | 99 |

LIST OF FIGURES

| | | |
|-----------|---|----|
| Figure 1 | Overview of proghrelin derived peptides (PGDP)..... | 4 |
| Figure 2 | Regulation of insulin secretion by glucose and the incretins..... | 23 |
| Figure 3 | MIN6, but not INS-1 cells express GHS R1a..... | 45 |
| Figure 4 | Acylated ghrelin (AG) and unacylated ghrelin (UAG) increase cell number in serum starved INS-1 cells | 48 |
| Figure 5 | Acylated ghrelin (AG), unacylated ghrelin (UAG) and obestatin (Ob) increase cell number in serum starved MIN6 cells | 50 |
| Figure 6 | Acylated ghrelin (AG), unacylated ghrelin (UAG) and obestatin (Ob) increase proliferation in serum starved INS-1 cells..... | 54 |
| Figure 7 | Acylated ghrelin (AG) and unacylated ghrelin (UAG) decrease apoptosis in palmitate treated INS-1 cells..... | 57 |
| Figure 8 | Acylated ghrelin (AG) decreases apoptosis in palmitate treated MIN6 cells..... | 60 |
| Figure 9 | Acylated ghrelin (AG) decreases caspase 3 activation in serum starved INS-1 cells..... | 62 |
| Figure 10 | Acylated ghrelin (AG) decreases caspase 3 activation in serum starved MIN6 cells..... | 65 |
| Figure 11 | Palmitate increases caspase 3 activation in INS-1 cells..... | 68 |
| Figure 12 | Acylated ghrelin (AG) and unacylated ghrelin (UAG) modestly increase phosphorylation of Akt in INS-1 cells..... | 70 |
| Figure 13 | Acylated ghrelin (AG) and unacylated ghrelin (UAG) modestly increase phosphorylation of Akt in MIN6 cells..... | 73 |
| Figure 14 | Acylated ghrelin (AG) regulates insulin secretion in INS-1 cells in a glucose and dose dependent manner | 75 |
| Figure 15 | Unacylated ghrelin (UAG) regulates insulin secretion in INS-1 cells in a glucose and dose dependent manner..... | 77 |
| Figure 16 | Obestatin (Ob) regulates insulin secretion in INS-1 cells in a glucose and dose dependent manner..... | 79 |

ABSTRACT

Proghrelin produces three proghrelin derived peptides (PGDP) known for their roles in glucose homeostasis: acylated ghrelin (AG), unacylated ghrelin (UAG), and obestatin (Ob). Only the receptor for AG, growth hormone secretagogue receptor 1a (GHS R1a), is known, however there is evidence for additional receptors for AG, UAG and Ob. Our aim was to determine the actions of PGDP on two beta cell lines, MIN6 and INS-1, which we have shown to contain and lack GHS R1a respectively. PGDP increased proliferation in INS-1 but not MIN6 cells, measured by BrdU incorporation. AG decreased apoptosis in both cell lines, measured by decreased levels of activated caspase 3. Insulin secretion was investigated in INS-1 cells, where PGDP modulated insulin release in a glucose dependent manner. Our results indicate that PGDP modulate beta cell mass in the presence and absence of GHS R1a, and present a detailed analysis of insulin secretion in INS-1 cells.

LIST OF ABBREVIATIONS USED

| | |
|---------------------|--|
| AC | Adenylyl Cyclase |
| ADP | Adenosine Diphosphate |
| AG | Acylated Ghrelin |
| AgRP | Agouti Related Peptide |
| AMPK | AMP-activated Protein Kinase |
| ANOVA | Analysis of Variance |
| Apaf1 | Apoptotic Protease Activating Factor 1 |
| ARC | Arcuate Nucleus |
| ATP | Adenosine triphosphate |
| BAD | Bcl-2-Associated Death Promoter |
| bp | Basepair |
| BrdU | 5-bromo-2'-deoxyuridine |
| BSA | Bovine Serum Albumin |
| Ca ⁺⁺ | Calcium Ions |
| cAMP | Cyclic Adenosine Monophosphate |
| cdk4 | Cyclin D-dependent Kinase 4 |
| cDNA | Complementary DNA |
| cGMP | Cyclic Guanosine Monophosphate |
| CRF2R | Corticotropin Releasing Factor 2 Receptor |
| dd H ₂ O | Purified Water |
| DNA | Deoxyribonucleic acid |
| DEPC | Diethylpyrocarbonate |
| EDTA | Ethylenediaminetetraacetic Acid |
| FBS | Fetal Bovine Serum |
| FFA | Free Fatty Acid |
| FoxO1 | Forkhead Box O1 |
| GH | Growth Hormone |
| GHRH | Growth Hormone Releasing Hormone |
| GHRP6 | Growth Hormone Releasing Peptide 6 |
| GHS | Growth Hormone Secretagogues |
| GHS R1a | Growth Hormone Secretagogue Receptor 1a |
| GIP | Glucose Dependent Insulinotropic Polypeptide |
| GLP-1 | Glucagon-like Peptide 1 |
| GLUT2 | Glucose Transporter 2 |
| GOAT | Ghrelin-O-Acyltransferase |
| GPR39 | G-protein Coupled Receptor 39 |
| GSIS | Glucose Stimulated Insulin Secretion |
| GSK3 | Glycogen Synthase Kinase 3 |
| GTT | Glucose Tolerance Test |
| HFD | High Fat Diet |
| IA-2 β | Insulinoma Associated Protein 2 beta |
| IGF-1 | Insulin-like Growth Factor 1 |
| IgG | Immunoglobulin G |

| | |
|------------------|---|
| ip | Intraperitoneal |
| K ⁺ | Potassium Ion |
| KO | Knock Out |
| MAPK | Mitogen Activated Protein Kinase |
| mRNA | Messenger RNA |
| mTORC1 | Mammalian Target of Rapamycin Complex 1 |
| mTORC2 | Mammalian Target of Rapamycin Complex 2 |
| nNOS | Neuronal Nitric Oxide Synthase |
| NO | Nitric Oxide |
| NPY | Neuropeptide Y |
| Ob | Obestatin |
| OxPhos | Oxidative Phosphorylation |
| PBS | Phosphate Buffered Saline |
| PC1 | Prohormone Convertase 1 |
| PC2 | Prohormone Convertase 2 |
| PCR | Polymerase Chain Reaction |
| PDK1 | Phosphoinositide-Dependent Kinase-1 |
| PFA | Paraformaldehyde |
| PGDP | Proghrelin Derived Peptides |
| PI3K | Phosphoinositide 3-Kinase |
| PIP3 | Phosphatidylinositol (3,4,5)-trisphosphate |
| PKA | Protein Kinase A |
| PKC | Protein Kinase C |
| PLC | Phospholipase C |
| PVDF | Polyvinylidene Fluoride |
| PVN | Paraventricular Nucleus |
| PTx | Pertussis Toxin |
| RIA | Radioimmunoassay |
| RNA | Ribonucleic Acid |
| RQ | Respiratory Quotient |
| rt PCR | Reverse Transcriptase Polymerase Chain Reaction |
| SDS-PAGE | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| SEM | Standard Error of the Mean |
| siRNA | Small Interfering RNA |
| sc | Subcutaneous |
| ScTx | Stromatoxin |
| T2D | Type 2 Diabetes |
| TEA | Tetraethyl Ammonium |
| UAG | Unacylated Ghrelin |
| UCP2 | Uncoupling Protein 2 |
| VG | Voltage Gated |
| wt | Wild Type |
| Zn ⁺⁺ | Zinc Ions |

ACKNOWLEDGEMENTS

I would like to acknowledge my supervisor, Dr. Younes Anini, for his support and guidance during the last 2 years as well as the other members of my supervisory committee, Dr. Paul Murphy and Dr. Ali Imran. I would also like to thank my fellow former and present lab colleagues, Dr. Micheal Morash, Angela MacDonald, Jeffrey Gagnon, Emily Sheppard and Kyungsoo Shin for their instruction and support. Finally I would like to thank Dr. Paul Murphy, Dr. Ali Imran, Dr. Kerry Goralski and Dr. Valerie Chappe for agreeing to serve as members of my examining committee.

CHAPTER 1 INTRODUCTION

1.1 GENERAL BACKGROUND

Ghrelin is a recently discovered peptide hormone, known for its stimulation of growth hormone (GH) secretion and food intake (Kojima et al., 1999). The identity of ghrelin was determined via reverse pharmacology (Kojima et al., 1999) following two decades of research on the G protein coupled receptor, growth hormone secretagogue receptor (GHS R1a) and a group of synthetic agonists, termed growth hormone secretagogues (GHS) (Howard et al., 1996). As such, the first known physiological role of ghrelin was the stimulation of GH release (Kojima et al., 1999). Shortly after ghrelin was discovered as the endogenous ligand of GHS R1a, reports followed of a potent ghrelin mediated stimulation of food intake in both rodents (Asakawa et al., 2001, Wren et al., 2001b, Tschop, Smiley and Heiman, 2000) and humans (Wren et al., 2001a). It is now clear that ghrelin is responsible for a diverse range of actions involved in the regulation of metabolism and energy homeostasis (Hassouna, Zizzari and Tolle, 2010, Kojima and Kangawa, 2008, Korbonits et al., 2004).

The concentration of endogenous ghrelin cycles with respect to feeding state; circulating ghrelin levels are increased during fasting and drop in response to re-feeding (Tschop, Smiley and Heiman, 2000, Guo et al., 2008). Ghrelin serum concentrations are also affected by long-term energy states, with ghrelin serum concentrations being decreased in obese individuals (Tschop et al., 2001, Erdmann et al., 2005) and increased in anorexic individuals (Otto et al., 2001, Broglio et al., 2004a). Likewise, circulating ghrelin is elevated in rodents undergoing chronic calorie restriction (Kinzig, Hargrave and Tao, 2009, Yang et al., 2007) or consuming a low protein diet (Lee et al., 2002),

while rodents fed a high fat diet (HFD) have a decreased concentration of circulating ghrelin (Lee et al., 2002). Furthermore, alterations in energy state are associated with modified ghrelin responses; obese humans have a less drastic decrease in circulating ghrelin after food intake as compared to normal weight individuals (Erdmann et al., 2005), while anorexia is associated with dampened GH secretion in response to ghrelin (Broglia et al., 2004a).

The actions of ghrelin however, extend beyond the stimulation of GH secretion and food intake. Treatment of rodents with exogenous ghrelin not only increases food intake (Wren et al., 2001b, Tschop, Smiley and Heiman, 2000, Currie et al., 2005, Nakazato et al., 2001), but also increases gut motility (Asakawa et al., 2001, Depoortere et al., 2008), alters adipocyte metabolism (Tschop, Smiley and Heiman, 2000, Theander-Carrillo et al., 2006, Thompson et al., 2004) and enhances the proliferation of cells in many tissues (Baldanzi et al., 2002, Camina et al., 2007, Granata et al., 2007, Chung et al., 2007, Johansson et al., 2008, Nanzer et al., 2004, Sato et al., 2006).

Ghrelin is predominantly produced in the stomach (Kojima et al., 1999), however other areas of the body contain ghrelin, albeit at much lower levels, including the small intestine, pancreas, adrenal glands, testes, fallopian tubes, cardiac tissue, ovaries, vasculature, kidney, liver, lung, adipose tissue, muscle, skin, spleen and thyroid gland (Lee et al., 2002, Gronberg et al., 2008, Wierup et al., 2004, Morash et al., 2010). As well, GHS R1a is found in a multitude of human and rat tissues including the hypothalamus, hippocampus, pituitary, thyroid, spleen, myocardium, adrenal glands and endocrine pancreas (Guan et al., 1997, Gnanapavan et al., 2002, McKee et al., 1997).

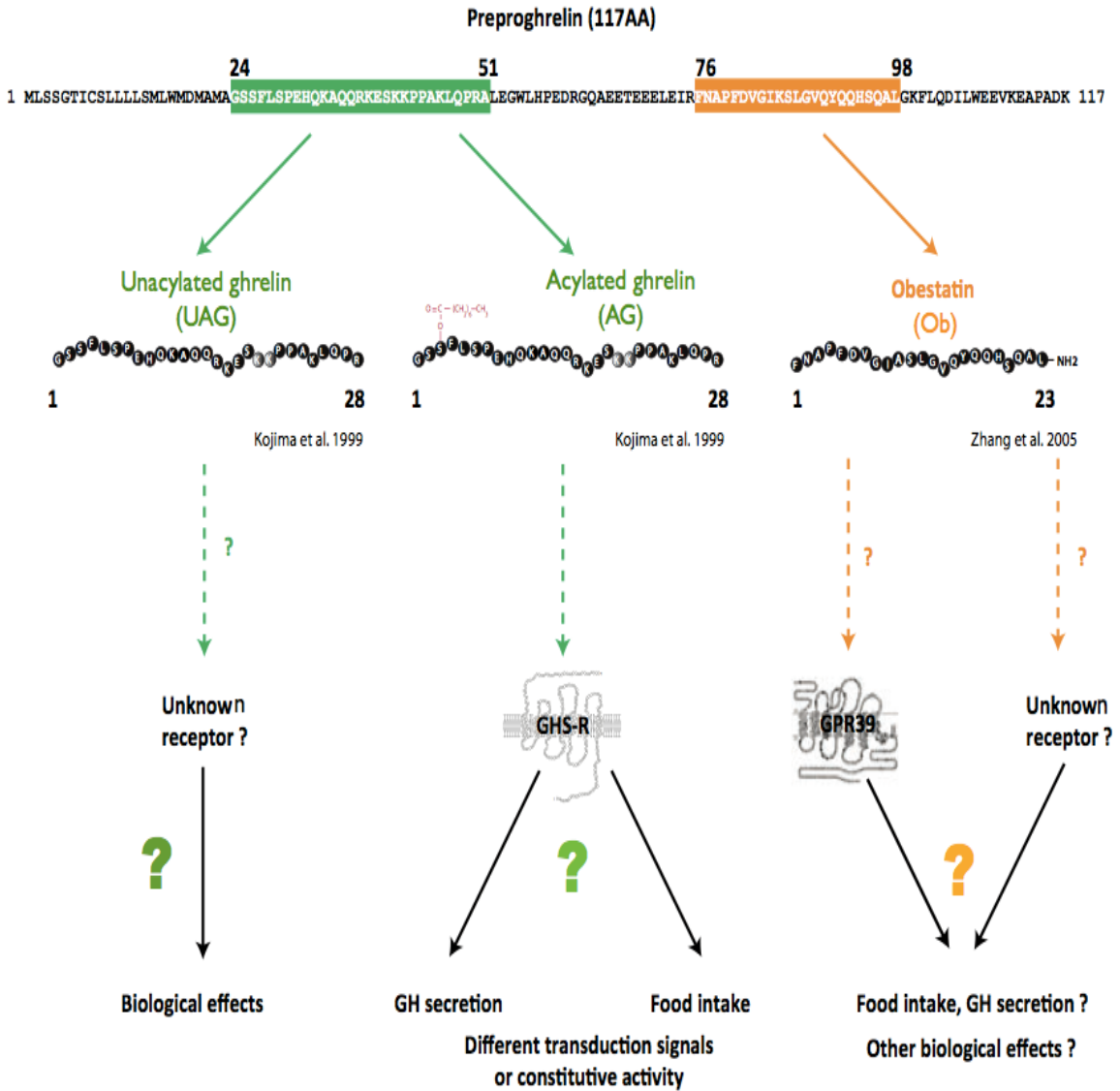
1.2 STRUCTURE AND PROCESSING OF PROGHRELIN

The ghrelin gene encodes a preproghrelin transcript that produces a preproghrelin peptide. Processing of preproghrelin can yield three different peptide hormones (proghrelin derived peptides (PGDP): 1) acylated ghrelin (AG), with an acyl modification on the third amino acid residue, serine 3, 2) unacylated ghrelin (UAG), which lacks this acyl modification, and 3) obestatin (Figure 1) (Asakawa et al., 2001, Hassouna, Zizzari and Tolle, 2010, Zhang et al., 2005). AG and UAG are derived from the N terminus of the proghrelin peptide. Ghrelin is cleaved from the proghrelin peptide by prohormone convertase 1 (PC1), as evidenced by the lack of mature ghrelin in PC1 knock out (KO) mice (Zhu et al., 2006). Furthermore, ghrelin positive cells in the stomach and pancreatic islets co-localize with PC1, confirming that ghrelin and PC1 are localized in the same cells (Zhu et al., 2006, Walia et al., 2008). Recently, other mechanisms of ghrelin processing have been suggested from experiments on COS-7 cells transfected with ghrelin in combination with various prohormone convertases. In addition to PC1, prohormone convertase 2 (PC2) and furin were shown to be sufficient for proghrelin processing, although the extent to which PC2 and furin contribute to proghrelin processing *in vivo* is not currently clear (Takahashi et al., 2009).

The acylation of ghrelin represents a unique modification that is not found on any currently known peptide hormone (Kojima et al., 1999). Ghrelin is acylated by the recently discovered enzyme Ghrelin-O-Acyltransferase (GOAT) (Yang et al., 2008, Gutierrez et al., 2008). The acylation of ghrelin seems to occur in the Golgi prior to processing of proghrelin as indicated by reports that proghrelin is acylated prior to

Figure 1. **Overview of proghrelin derived peptides (PGDP).** Acylated ghrelin (AG), unacylated ghrelin (UAG) and obestatin (Ob) are derived from the proghrelin peptide. AG differs from UAG by an acyl modification on the 3rd amino acid residue, serine 3 by the enzyme Ghrelin-O-Acyltransferase (GOAT). This post-translational modification enables AG to bind to and activate GHS R1a, through which AG stimulates growth hormone (GH) secretion and increases food intake. UAG is not able to bind to GHS R1a. However, UAG has been reported to stimulate proliferation and inhibit apoptosis in a variety of tissues and cell lines indicating that there is a currently unidentified receptor for UAG. Furthermore, AG has been reported to have actions in tissues and cell lines lacking GHS R1a, indicating that there is an unidentified receptor for AG as well. Ob was originally reported to activate GPR39, however these findings are highly controversial and the identity of the receptor for Ob is also considered unknown. (figure has been modified from Hassouna, Zizzari and Tolle, 2010).

Proghrelin Derived Peptides



Modified from (Hassouna et al. 2010)

Figure 1

cleavage into mature ghrelin (Zhu et al., 2006, Yang et al., 2008). The distribution of GOAT closely resembles that of ghrelin (Morash et al., 2010, Yang et al., 2008, Gutierrez et al., 2008, Gonzalez et al., 2008). The short term metabolic regulation of GOAT expression is unclear, with reports that gastric GOAT expression is not sensitive to 24 or 48 hour fast (Morash et al., 2010, Gonzalez et al., 2008), and other reports that GOAT expression declines with a similar period of fasting (Kirchner et al., 2009). However, mice with a chronically positive or negative energy balance (achieved by dietary excess or restriction) have elevated GOAT expression (Morash et al., 2010, Gonzalez et al., 2008). Interestingly, in *ob/ob* mice, a model of obesity due to leptin deficiency, GOAT expression is greatly reduced, indicating that leptin levels may regulate the expression of this enzyme (Morash et al., 2010). It has also been shown that leptin injections in fasted rats increase GOAT expression in the stomach (Gonzalez et al., 2008). Together, these reports suggest that GOAT levels may be regulated by leptin. However, an understanding of the factors that control the rates of ghrelin acylation remains incomplete.

Obestatin is derived from the C terminus of proghrelin (Zhang et al., 2005) and the enzyme responsible for its cleavage from proghrelin is currently unknown.

Obestatin shares a similar distribution to that of ghrelin (Walia et al., 2008), with immunopositive cells in the stomach, small intestine and pancreas. Furthermore, co-localization of ghrelin with obestatin has been reported in human gastric mucosa, proximal regions of the small intestine, mammary glands and around the periphery of pancreatic islets (Gronberg et al., 2008).

1.3 RECEPTORS FOR PROGHRELIN DERIVED PEPTIDES BEYOND GHS R1A

1.3.1 Unacylated Ghrelin is Not a Ligand for GHS R1a

AG, but not UAG, binds to the GHS R1a, and is responsible for the stimulation of GH release and food intake by ghrelin (Asakawa et al., 2001, Bednarek et al., 2000, Bednarek et al., 2000, Sun et al., 2004, Hosoda et al., 2000) (Figure 1). However, UAG is the major circulating form of ghrelin, as the fraction of AG makes up only approximately 2-10% of total circulating ghrelin (Hosoda et al., 2000, Morash et al., 2010). UAG was originally reported to lack bioactivity (Kojima et al., 1999), but this idea has been challenged by other groups (Granata et al., 2007, Chen et al., 2005, Toshinai et al., 2006). Central administration of UAG has been shown to induce food intake in GHS R1a KO mice (Toshinai et al., 2006), via activation of an unknown receptor on orexin neurons in the lateral hypothalamus. However, others have reported systemic and central UAG treatment to decrease food intake and decrease gastric motility (Chen et al., 2005, Asakawa et al., 2005). The receptor for UAG is currently unknown (Camina, 2006, Soares and Leite-Moreira, 2008) (Figure 1).

1.3.2 Evidence for Unknown Receptors for Acylated Ghrelin

The receptor(s) for ghrelin require further investigation, as the possibility of additional receptors for AG has been suggested (Camina, 2006, Muccioli et al., 2007). Activities of AG have been reported in cells lacking GHS R1a, indicating that AG signals through at least one unknown receptor. For example, AG stimulates proliferation and inhibits apoptosis in rat hippocampal progenitor cells, which do not express GHS R1a. These cells exhibit binding sites specific for AG and the GHS, hexarelin, but not UAG (Johansson et al., 2008); the identity of this receptor remains unknown.

There is also abundant evidence that there is an unknown receptor that binds both AG and UAG. The hamster pancreatic beta cell line, HIT-T15, responds to AG with increased insulin secretion, proliferation and decreased apoptotic activity despite lacking GHS R1a (Granata et al., 2007). HIT-T15 cells also have binding sites that share specificity for both AG and UAG, as indicated by competitive radioligand binding experiments (Granata et al., 2007). The receptor in HIT-T15 cells that recognizes AG and UAG remains unknown. Further evidence for these unknown ghrelin receptors comes from experiments using antibodies against AG or UAG in cultured human erythroleukemic cells. These cells lack GHS R1a, and antibodies against AG or UAG inhibit proliferation, again suggesting that there exists an additional receptor to GHS R1a that recognizes both AG and UAG (De Vriese et al., 2005). Such a receptor has also been suggested in H9c2 cardiomyocytes, which also lack GHS R1a and possess a common binding site for AG and UAG (Baldanzi et al., 2002). Likewise, AG and UAG stimulate proliferation in a human osteoblast cell line, SV-HFO, which lacks GHS R1a (Delhanty et al., 2006). AG and UAG also compete for specific binding sites in rat fetal spinal cord tissues, with both AG and UAG increasing proliferation (Sato et al., 2006). Breast cancer cells have been shown to lack GHS R1a, and have specific binding sites that recognize GHS, AG and UAG. In these cells, GHS, AG and UAG induce proliferation - further suggesting an unknown receptor that recognizes GHS, AG and UAG (Cassoni et al., 2001). Similar binding sites appear to be present in prostate cancer cells as well (Cassoni et al., 2004), although ghrelin inhibits proliferation in these cells.

The results from these studies indicate that there exist two additional unknown receptors for AG, one which is also capable of binding UAG (Granata et al., 2007, De

Vriese et al., 2005, Baldanzi et al., 2002, Delhanty et al., 2006, Sato et al., 2006, Cassoni et al., 2001) and one which is not (Johansson et al., 2008). The identification of these receptors is essential for a full understanding of ghrelin.

1.3.3 Unacylated Ghrelin Receptor

The existence of a receptor that recognizes UAG but not AG is suggested by recent results in human cardiomyocytes. These cells have been shown to possess two independent UAG binding sites that do not recognize AG (Lear et al., 2010).

Furthermore, although AG and UAG inhibited apoptosis in these cells, AG and UAG had different effects on cellular metabolism, with AG inhibiting insulin stimulated glucose uptake, and UAG stimulating the uptake of fatty acids (Lear et al., 2010). Corticotropin releasing factor 2 receptor (CRF2R) was originally suggested as a possible receptor for UAG after reports that blockade of CRF2R inhibits the negative effects of UAG on gastric motility (Chen et al., 2005). However, UAG is also reported to stimulate insulin secretion in INS-1E cells which lack CRF2R expression (Gauna et al., 2006).

Furthermore, CRF2R antagonists do not block the actions of UAG in this cell line, suggesting that the actions of UAG can occur independently of CRF2R or other receptors that recognize CRF2R antagonists (Gauna et al., 2006). Surprisingly, in cells transfected with human GHS R1a, supra-physiological doses of UAG (μM concentrations) stimulates intracellular Ca^{++} levels via GHS R1a, indicating that UAG is capable of stimulating GHS R1a (Gauna et al., 2007). This however, does not account for the effects seen in cells lacking GHS R1a (Baldanzi et al., 2002, Granata et al., 2007, De Vriese et al., 2005, Delhanty et al., 2006). The identity of potential UAG receptors remains unknown, as

does an understanding of the ability and circumstances under which UAG can bind to CRF2R and GHS R1a.

1.3.4 Obestatin Receptor

Obestatin was originally reported to oppose the actions of AG via signaling through G-protein coupled receptor 39 (GPR39) (Zhang et al., 2005), causing decreased food intake and reduced weight gain (Zhang et al., 2005, Green, Irwin and Flatt, 2007). However, both the actions of obestatin and the identity of its receptor are controversial, with many groups failing to replicate the original findings (Depoortere et al., 2008, Unniappan, Speck and Kieffer, 2008, Tang et al., 2008, Lauwers et al., 2006, Kobelt et al., 2008a). It has been suggested that the supposed obestatin activation of GPR39 is due to Zn⁺⁺ contamination in obestatin peptide preparations (Lauwers et al., 2006, Holst et al., 2007). In response to these claims, Zhang et al. (2008a), the original group that discovered obestatin, confirmed their original findings of obestatin binding to GPR39, suggesting that the location of the iodination of the peptide is critical to maintain proper binding activity in radioligand binding assays. Furthermore, Zhang et al. has shown that acute obestatin treatment in mice induces the expression of the immediate early gene c-fos in the gastric mucosa, intestinal villi and adipose cells, suggesting that obestatin does elicit biological actions *in vivo* (Zhang et al., 2008a). To date, the actions of obestatin and the identity of the receptor for this hormone remain contentious and require more investigation.

1.3.5 Further Controversies on Obestatin's Receptor

Given the controversial status of GPR39 as the receptor for obestatin (Lauwers et al., 2006, Holst et al., 2007, Zhang et al., 2008a), there exists a strong possibility that the actions of obestatin are not mediated through GPR39 (Lauwers et al., 2006, Holst et al., 2007). Recently, obestatin has been shown to interact with a variety of other hormonal receptors. It was suggested that obestatin may interact with the glucagon-like peptide 1 (GLP-1) receptor, as well as binding sites representing an unknown AG/UAG receptor in HIT-T15 cells (Granata et al., 2008). As well, the proliferative and anti-apoptotic action of obestatin in the pancreatic beta cell line, INS-1E, was blocked with co-treatment with anti-ghrelin antibody (Granata et al., 2008), indicating that the presence of ghrelin is important for the bioactivity of obestatin in this model. Application of a GLP-1 receptor antagonist also serves to block the pro-survival effects of obestatin in INS-1E cells (Granata et al., 2008), indicating that obestatin may interact with GLP-1 receptor. However, another group has reported that obestatin exhibits no binding to HEK cells transfected with GLP-1 receptor (Unniappan, Speck and Kieffer, 2008). The discrepancies in obestatin binding may be due to the interactions of GPR39 with additional receptors, for instance obestatin was recently shown to activate Akt signaling the gastric cell line, KATO- III, via interactions between GPR39, β arrestin and epidermal growth factor receptor (Alvarez et al., 2009).

1.3.6 Reports of GHS R1a Receptor Dimerization

Similarly to GPR39, GHS R1a activity may be modulated by its interactions with additional receptors. The complete functioning of GHS R1a, along with its modulation by dimerization is currently unknown, but may explain the variations seen in ghrelin

responsiveness in various cells. GHS R1a has been suggested to dimerize with other G protein coupled receptors, mainly dopamine receptor subtype 1 and growth hormone releasing hormone (GHRH) receptor, as ghrelin in these systems causes a potentiation of cyclic adenosine monophosphate (cAMP) signaling with co-treatment with dopamine or GHRH that is not inducible by ghrelin alone (Jiang, Betancourt and Smith, 2006, Kineman and Luque, 2007, Cunha and Mayo, 2002).

Clearly, a further understanding in the dimerization and altered functioning of GHS R1a, along with the identities of the unknown, but postulated AG, UAG and obestatin receptors is of utmost importance for understanding the actions of PGDP and may lead to a resolution of some of the controversies on ghrelin action.

1.4 RECEPTOR AND SIGNALING PATHWAY IN ACYLATED GHRELIN STIMULATED GH SECRETION

The first reports of the biological action of ghrelin were of stimulation of GH secretion by the activation of the GHS R1a (Kojima et al., 1999), following the known actions of synthetic GHS. Therefore, the prototypical signaling mechanism for GHS R1a was elucidated in somatotrophs (Malagon et al., 2003). The mechanism for GH release via GHS R1a activation is a result of increased phospholipase C (PLC) / protein kinase C (PKC) signaling, opening of voltage gated Ca^{++} channels, leading to increased intracellular Ca^{++} and GH secretion (Kineman and Luque, 2007, Malagon et al., 2003). Activation of adenylyl cyclase (AC)/ protein kinase A (PKA) signaling has also been shown in porcine somatotrophs in response to ghrelin in the absence of GHRH (Malagon et al., 2003). However, as mentioned previously, other models have shown GHS R1a activation only increases cAMP in the presence of GHRH, an effect that is synergistic,

suggesting that there may be an interaction between GHS R1a and GHRH receptor (Malagon et al., 2003, Han et al., 2005). Recently, the involvement of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) signaling in the stimulation of GH release by ghrelin has also been indicated (Han et al., 2005, Rodriguez-Pacheco et al., 2005, Rodriguez-Pacheco et al., 2008).

1.5 GHRELIN AND FOOD INTAKE

The most well studied effect of ghrelin, however, is stimulation of food intake and regulation of body weight by AG. Both peripheral and central administration of exogenous ghrelin increases food intake (Wren et al., 2001b, Tschop, Smiley and Heiman, 2000, Currie et al., 2005, Nakazato et al., 2001). Furthermore, GHS R1a antagonists decrease food intake when given both systemically or centrally (Asakawa et al., 2003). Mice receiving daily injections of ghrelin concurrently with a HFD, show increased adiposity compared to control mice receiving saline injection and eating HFD (Asakawa et al., 2003). Chronic administration of GHS R1a agonist, BIM-28131, in rats via subcutaneous (sc) mini pumps results in increased body weight and fat mass, as well as increased food intake (Strassburg et al., 2008). The administration of a catalytic ghrelin antibody that hydrolyzes AG to UAG increases metabolic rate in mice, as evidenced by increased heat production and oxygen consumption, and decreases food intake during re-feeding in response to a 24 hour fast (Mayorov et al., 2008).

Ghrelin stimulation of food intake occurs in the hypothalamus, where there are a small population of neurons which express ghrelin, located adjacent to the arcuate (ARC), and dorsal, ventral and paraventricular nuclei (PVN) (Cowley et al., 2003, Mondal et al., 2005). Hypothalamic ghrelin expression and content decrease in response

to fasting, with this decrease in hypothalamic ghrelin peptide most likely due to increased ghrelin secretion (Sato et al., 2005). Despite the central production of ghrelin, peripheral injections of ghrelin can also activate the feeding centers of the brain (Kobelt et al., 2008b). Peripheral ghrelin crosses the blood brain barrier using saturable transporters (Banks et al., 2002), the activity of which are enhanced with decreased body weight (Banks, Burney and Robinson, 2008). As such, fasting rodents not only have increased circulating ghrelin (Kinzig, Hargrave and Tao, 2009), but also have enhanced transport of ghrelin across the blood brain barrier (Banks, Burney and Robinson, 2008).

Ghrelin induces food intake by its actions in the ARC. Ghrelin signals via GHS R1a to directly stimulate orexigenic neuropeptide Y (NPY)/ Agouti related peptide (AgRP) neurons and indirectly inhibit anorexigenic prepro-melanocortin neurons (Cowley et al., 2003, Mondal et al., 2005, Kohno et al., 2003). Despite activating GHS R1a, AG stimulates different signaling pathways in the hypothalamus as compared to the pituitary. The stimulatory effects of ghrelin on NPY/AgRP neurons have recently been shown to occur by ghrelin activation of mitochondrial respiration mediated by the AMP-activated protein kinase (AMPK)/ uncoupling protein 2 (UCP2) pathway, which leads to increased fatty acid beta oxidation and neutralization of the produced reactive oxygen species, enabling sustained activity of the neuron (Andrews et al., 2008, Kohno et al., 2008).

1.6 ANIMAL MODELS OF GHRELIN ACTION

1.6.1 PGDP Axis KO Models

Many animal models have been created to examine the physiological effects of PGDP, including gene knock outs of either PGDP, GOAT, GHS R1a or the proposed

obestatin receptor GPR39 (Depoortere et al., 2008, Kirchner et al., 2009, Sun et al., 2004, Tremblay et al., 2007, Shuto et al., 2002, Zigman et al., 2005, Wortley et al., 2005, De Smet et al., 2006, Sun, Ahmed and Smith, 2003, Sun et al., 2008, Sun et al., 2006, Pfluger et al., 2008, Moechars et al., 2006). However, the results produced by deleting proteins from the ghrelin axis have yielded weak phenotypes. Far from the expected phenotype of reduced growth and food consumption, ghrelin, GOAT and GHS R1a KO animals exhibit a modest phenotype (Kirchner et al., 2009, Sun et al., 2004, De Smet et al., 2006, Sun, Ahmed and Smith, 2003, Sun et al., 2008) displaying deficiencies in body weight and food intake only when challenged by a HFD (Kirchner et al., 2009, Zigman et al., 2005, Wortley et al., 2005). This is most likely due to compensatory systems in these KO mice, given the importance of body weight regulation and maintenance of energy stores in organisms (De Smet et al., 2006, Sun, Ahmed and Smith, 2003, Sun et al., 2008). Despite these mild effects of ghrelin or GHS R1a deletion, the confirmation that AG acts via GHS R1a to stimulate GH secretion and food intake, came from GHS R1a KO mice, which lack a GH or feeding response to AG injections (Sun et al., 2004).

Subtle differences in ghrelin KO mice versus wild type (wt) mice do exist; young KO mice have a decreased respiratory quotient (RQ), coupled with enhanced heat production indicating that KO mice have alterations to their metabolism (De Smet et al., 2006). Ghrelin and GHS R1a KO mice also show subtle differences compared to wt animals, such as lowered blood glucose (Zigman et al., 2005, Sun et al., 2008, Longo et al., 2008). Ghrelin KO mice have enhanced glucose stimulated insulin secretion (GSIS); glucose tolerance tests (GTT) show increased insulin response and a resultant faster clearance of glucose from circulation (Dezaki et al., 2006). Furthermore, when fed HFD,

ghrelin KO mice were resistant to HFD induced glucose intolerance (Dezaki et al., 2006). Interestingly, hyperglycemia and peripheral insulin sensitivity is also improved when obese *ob/ob* mice are crossed with ghrelin KO mice, despite no change in body weight, suggesting that modulating glucose homeostasis may be an important role of ghrelin (Sun et al., 2006).

Recently, mice that lack both ghrelin and GHS R1a have been generated, and display a stronger phenotype than single KO mice, with decreased body weight and increased energy expenditure under normal feeding regimens (Pfluger et al., 2008). As well, ghrelin KO mice have also been treated with obestatin to determine if the deletion of obestatin concurrently with ghrelin is responsible for the lack of phenotype in ghrelin KO mice (Depoortere et al., 2008); however, obestatin treatment was not successful in uncovering a metabolic phenotype in these mice. In agreement with this finding, deletion of the putative obestatin receptor, GPR39, results in mice without altered body weight or food intake (Tremblay et al., 2007), however other groups have reported physiological effects of GPR39 deletion, with reports of increased gastric motility, body weight, cholesterol, fat mass, (Moechars et al., 2006) and dampened insulin response to both oral and intravenous glucose challenge (Holst et al., 2009, Tremblay et al., 2009).

1.6.2 Additional Approaches to Animal Models of Reduction or Excess of PGDP

Attempts to avoid developmental compensation for lack of ghrelin signaling have been taken using partial knock down models. Transgenic rats with GHS R1a knocked down in the ARC have lowered body weight and fat mass, along with reduced food intake (Shuto et al., 2002), a phenotype that is more pronounced than rodents with a

global deletion of GHS R1a. Furthermore, transgenic mice overexpressing both ghrelin and GOAT have increased body weight and fat mass along with decreased energy expenditure, despite their normal volume of food consumption (Kirchner et al., 2009). Transgenic mice globally overexpressing UAG were reported to have decreased body weight, fat mass, gastric motility and food intake (Asakawa et al., 2005). Another group has probed the actions of UAG on adiposity by overexpressing UAG in mouse adipose tissue, finding that overexpression of UAG in adipose decreased fat mass and provided a resistance to weight gain on a HFD, as well as improved insulin sensitivity in peripheral tissues (Zhang et al., 2008b).

Decreasing the endogenous ghrelin by giving rats a central infusion of anti-ghrelin IgG, which immunoneutralizes endogenous ghrelin, decreases food intake (Nakazato et al., 2001, Bagnasco et al., 2003). Other methods of decreasing AG levels *in vivo* have also been reported (Shearman et al., 2006, Zorrilla et al., 2006). Neutralization of AG using RNA spiegelmer results in decreased food intake and weight loss in mice (Shearman et al., 2006), while AG neutralization via ghrelin immunoconjugates results in slowed weight gain due to decreased feed efficiency (Zorrilla et al., 2006). Recently, our lab has shown that introduction of a truncated GHS R1a into the circulation of mice, designed to neutralize circulating AG, decreases fat stores and increases performance on glucose tolerance tests (GTT) (unpublished data).

1.7 GHRELIN AND GLUCOSE HOMEOSTASIS

Apart from the effect of ghrelin on GH release and food intake, KO models have suggested a role for ghrelin in glucose homeostasis as evidenced from reports of KO models with improved blood glucose regulation (Zigman et al., 2005, Sun et al., 2008,

Sun et al., 2006, Longo et al., 2008, Dezaki et al., 2006, Zhang et al., 2008b). In humans, administration of AG to healthy individuals causes a decline in circulating insulin and subsequently an increase in blood glucose (Broglia et al., 2001, Broglia et al., 2004b). Alterations in the ghrelin axis are also seen in humans with type 2 diabetes (T2D) who have decreased serum levels of ghrelin (Erdmann et al., 2005, Poykko et al., 2003). Administration of a GHS R1a antagonist in both normal weight or obese rats (Esler et al., 2007) or mice (Dezaki et al., 2006) improves insulin response to GTT, clearing glucose from the blood more efficiently than in control rodents. As such the ghrelin axis appears to modulate insulin release and sensitivity, however, the exact relationship between ghrelin and glucose homeostasis is unknown.

PGDP regulation of insulin secretion has been controversial, with reports of both stimulation and inhibition of insulin secretion for AG (stimulation (Lee et al., 2002, Granata et al., 2007, Gauna et al., 2006, Date et al., 2002, Adeghate and Ponery, 2002, Salehi et al., 2004, Qader et al., 2008) and inhibition (Wierup et al., 2004, Dezaki et al., 2006, Esler et al., 2007, Salehi et al., 2004, Qader et al., 2008, Reimer, Pacini and Ahren, 2003, Qader et al., 2005, Dezaki et al., 2004, Dezaki, Kakei and Yada, 2007, Colombo et al., 2003, Wang et al., 2010c)) and obestatin (stimulation (Granata et al., 2008, Egido et al., 2009), inhibition (Qader et al., 2008, Egido et al., 2009, Ren et al., 2008), and no effect (Unniappan, Speck and Kieffer, 2008)). UAG has been reported to have either no effect (Dezaki et al., 2006, Esler et al., 2007, Qader et al., 2008), or to stimulate insulin secretion (Granata et al., 2007, Gauna et al., 2006). Furthermore, although AG is speculated to act via GHS R1a in the pancreas, effects of AG on insulin secretion have been found in HIT-T15 cells, a pancreatic beta cell line that does not express GHS R1a

(Granata et al., 2007). Given these discrepancies, more work is needed to determine the actions of PGDP in the endocrine pancreas.

1.7.1 Affect of PGDP on Insulin Secretion *In Vivo*

In vivo, ghrelin administration has been reported to stimulate (Lee et al., 2002) or inhibit (Dezaki et al., 2006, Salehi et al., 2004, Dezaki et al., 2004, Dezaki, Kakei and Yada, 2007) insulin secretion. Inhibitory action of ghrelin on insulin secretion may be due to the activation of GHS R1a, as rats treated with a GHS R1a antagonist have elevated concentrations of circulating insulin (Dezaki et al., 2004, Dezaki, Kakei and Yada, 2007). Antagonism of GHS R1a in rodents (Esler et al., 2007, Dezaki et al., 2006) improves insulin response to GTT, resulting in a reduction of blood glucose as compared to control animals, suggesting that blockade of GHS R1a signaling may be an effective treatment for T2D. However, given the discrepancies between these results, more work is needed to gather a complete understanding of how insulin stimulation and inhibition can be achieved with ghrelin. As well, a full understanding of the actions of ghrelin in systems lacking GHS R1a is needed.

Leptin deficient *ob/ob* mice display decreased blood glucose when given daily intraperitoneal (ip) injections of a GHS R1a antagonist (Asakawa et al., 2003). Furthermore, *ob/ob* mice crossed with ghrelin KO mice have enhanced insulin secretion, and show protection from glucose intolerance when fed a HFD (Dezaki et al., 2006). GHS R1a KO mice also have decreased circulating glucose and insulin, and improved insulin sensitivity (Longo et al., 2008), further indicating that blockade of the ghrelin axis may be a beneficial treatment for diabetes. The relationship between ghrelin and glucose homeostasis appears to be due to the direct action of ghrelin on insulin secretion from

pancreatic beta cells. Indeed, antagonism of GHS R1a in gastrectomized rats, which lack the organ that is the predominant source of systemic ghrelin, still increases insulin secretion (Dezaki et al., 2006), suggesting that a local release of ghrelin in the pancreas is sufficient for ghrelin regulation of insulin release.

1.7.2 Pancreatic Ghrelin

Further supporting a direct role of ghrelin on pancreatic beta cells is the discovery of pancreatic ghrelin cells. Ghrelin peptide is present in the pancreatic islets, however the identity of the cell type is controversial, with reports of ghrelin co-localizing with alpha cells (Date et al., 2002), beta cells (Volante et al., 2002) or in novel and separate secretory cells termed epsilon cells (Wierup et al., 2004, Wierup et al., 2002, Wierup and Sundler, 2005). Epsilon cells have been identified in both rodent (Wierup et al., 2004) and human (Wierup et al., 2002, Wierup and Sundler, 2005) islets. The identity of the ghrelin cells has also been reported to vary with age, with ghrelin cells co-localizing with glucagon or pancreatic polypeptide in neonate rodents, but belonging to the separate endocrine epsilon cells in adult rats (Wierup et al., 2004). In human islets, ghrelin and obestatin positive cells have been detected in a low number of cells in the periphery of adult human islets (Gronberg et al., 2008). As well, another group has reported that ghrelin immunopositive cells co-localize with alpha cells at the periphery of adult human and rodent islets (Date et al., 2002).

The expression of ghrelin, as well as GHS R1a was confirmed in rat islets via PCR (Date et al., 2002, Chanoine and Wong, 2004). Furthermore, the concentration of both AG and UAG has been found to be higher in the pancreatic veins than the pancreatic arteries (8 fold and 3 fold increase respectively) (Dezaki et al., 2006), suggesting that

ghrelin is not only present, but is secreted from cells within the pancreas.

Immunostaining of human pancreatic islets, shows that GHS R1a is present in a portion of beta cells (Granata et al., 2007). Mouse and rat islets have also have a portion of beta cells that co-localize with the controversial obestatin receptor GPR39 (Unniappan, Speck and Kieffer, 2008). As such, both PGDP and their identified receptors are present in pancreatic islets. Antagonism of GHS R1a (Dezaki et al., 2006, Dezaki, Kakei and Yada, 2007) enhances GSIS in rats *in vivo*, with similar results in a perfused pancreas model following immunoneutralization of endogenous ghrelin (Dezaki et al., 2006). Together, these results suggest that ghrelin within pancreatic islets is able to modulate beta cell function, via a paracrine mechanism.

The quantity of pancreatic ghrelin cells is age dependent, being numerous in the fetal pancreas and decreasing from birth onwards to the adult phenotype of 0-2 ghrelin positive cells per islet (Wierup et al., 2004, Walia et al., 2008, Wierup et al., 2002). In fact, in fetal tissues, the ghrelin content of the pancreas is far greater than that of the stomach, the main source of ghrelin in adults (Kojima et al., 1999). This high level of fetal pancreatic ghrelin suggests ghrelin may play an important role in islet development (Chanoine and Wong, 2004, Nakahara et al., 2006). Other pancreatic PGDP protein levels also fluctuate with respect to age, with UAG peaking in the days prior to birth, followed by a gradual decline up until weaning, as compared to AG which peaks one week after birth (Chanoine, Wong and Barrios, 2006). Interestingly, the distribution of pancreatic ghrelin cells has been shown to be altered with diabetes; non-diabetic rat islets contain few, peripheral ghrelin positive cells, while islets from rats with streptozotocin

induced diabetes have more ghrelin positive cells which furthermore, are atypically located within the central region of islets (Adeghate and Ponery, 2002).

1.7.3 Molecular Regulation of Insulin Secretion

Glucose is the prototypical stimulator of insulin secretion from beta cells. Glucose enters the beta cell via non-insulin dependent glucose transporter 2 (GLUT-2) where it proceeds to enhance the output from the glycolytic pathway. The resulting increase in the ATP/ADP ratio, leads to closure of the K^+ ATP channel, causing the depolarization of the cell and the consequent opening of voltage gated (VG) Ca^{++} channels and subsequent influx of Ca^{++} . The increase in intracellular Ca^{++} triggers exocytosis of insulin secretory granules (Figure 2) (MacDonald, Joseph and Rorsman, 2005, Maechler, Carobbio and Rubi, 2006).

In addition to ghrelin, other gastrointestinal peptide hormones play a role in regulation of insulin secretion, most notably the incretins: GLP-1 and glucose dependent insulinotropic polypeptide (GIP). Interestingly, the secretion of these hormones is also regulated by feeding state, however, unlike ghrelin, GLP-1 and GIP secretion is triggered by food intake, not by fasting. Both GLP-1 and GIP stimulate insulin secretion in a glucose dependent manner via cAMP/PKA signaling (Figure 2), and enhance beta cell proliferation and survival via phosphoinositide 3-kinase (PI3K)/Akt signaling (Baggio and Drucker, 2007, Doyle and Egan, 2007).

The effects of ghrelin on insulin secretion appear to be mediated through a distinct signaling pathway from glucose and the incretins. Furthermore, the signaling mechanism for ghrelin modulation of insulin secretion is different from the pathway by which ghrelin stimulates GH secretion. Blockade of the G-protein subunits subtype

Figure 2. **Regulation of insulin secretion by glucose and the incretins.** Beta cells secrete insulin after stimulation by glucose. Metabolized glucose results in an increased ATP/ADP ratio which closes K^+ ATP channels, depolarizing the beta cell. This change in membrane potential opens voltage gated (VG) Ca^{++} channels. The resultant rise in intracellular Ca^{++} triggers the exocytosis of insulin granules. The incretins GLP-1 and GIP act to potentiate the response to glucose by mobilizing intracellular Ca^{++} stores via cAMP/PKA signaling. Both the mechanism and receptor through which PGDP effect insulin secretion is currently unknown.

Regulation of Insulin Secretion By Glucose and the Incretins

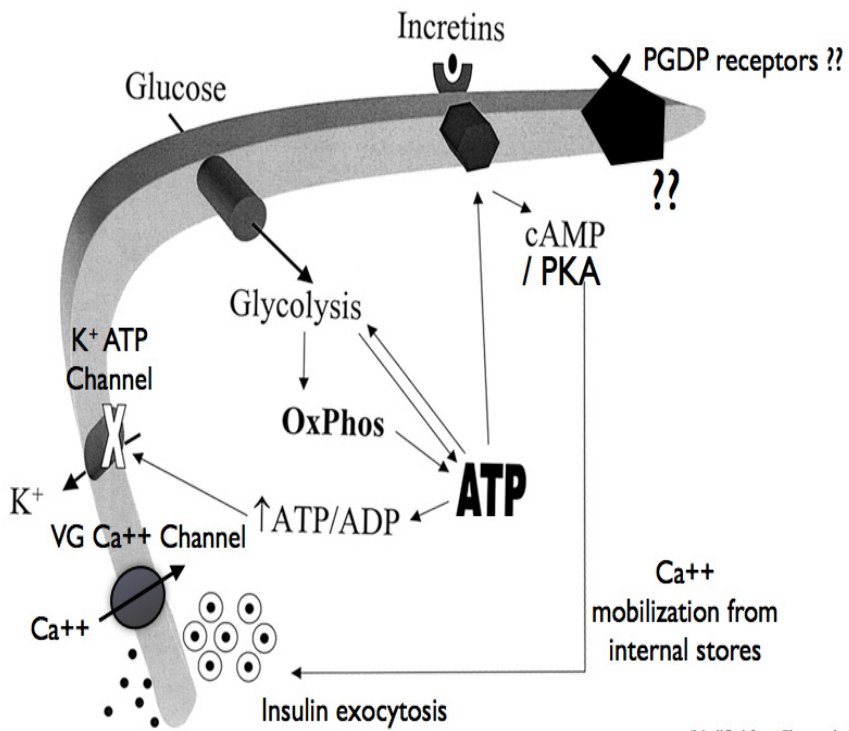


Figure 2

$G\alpha_{i/o}$, with pertussis toxin (PTx), abolishes the inhibitory actions of ghrelin on insulin secretion in rats both *in vivo* and in rat islet preparations, while PTx had no effect on ghrelin induced GH secretion (Dezaki, Kakei and Yada, 2007), suggesting that GHS R1a is coupled to different signaling pathways in somatotrophs versus pancreatic beta cells. GHS R1a antagonism increases insulin secretion from rat islets, while application of ghrelin decreases insulin secretion, an effect which is blocked with PTx (Dezaki, Kakei and Yada, 2007). Ghrelin treatment increases outward K^+ current through voltage dependent K^+ channels (Kv) in isolated rat beta cells (Dezaki, Kakei and Yada, 2007, Dezaki, Sone and Yada, 2008), specifically the Kv2.1 channel, as both inhibition of Kv channels (tetraethyl ammonium (TEA)) and specific Kv2.1 channel inhibitor (stromatoxin (ScTx)) block ghrelin suppression of insulin secretion. Ghrelin decreases the frequency and amplitude of glucose stimulated action potential in beta cells, as well as the glucose mediated rise in intracellular Ca^{++} levels via a $G\alpha_i$ signaling mechanism (Dezaki, Kakei and Yada, 2007). PTx or presence of a cAMP analog blocked ghrelin enhanced Kv channel currents, suggesting $G\alpha_i$ and cAMP signaling lie upstream of Kv2.1 channel. Knockdown of G-protein, $G\alpha_{i2}$, via siRNA, also blocks ghrelin induced suppression of insulin secretion (Dezaki, Kakei and Yada, 2007). Other groups have shown that ghrelin causes up-regulation of insulinoma-associated protein 2 beta (IA-2 β), a glycoprotein localized to secretory vesicles, in MIN6 insulinoma cells, and that IA-2 β expression is necessary for the inhibitory actions of ghrelin on insulin secretion, suggesting that ghrelin may also act to inhibit insulin secretion at the level of exocytosis (Doi et al., 2006). Ghrelin also increases UCP2 expression via AMPK activation in MIN6 cells, with siRNA against UCP2, blocking the inhibitory actions of ghrelin on insulin

secretion (Wang et al., 2010c). Ghrelin has also been shown to inhibit insulin secretion via enhanced NO production by constitutive neuronal nitric oxide synthase (nNOS) (Qader et al., 2005).

In models where AG stimulates insulin secretion, the enhancement of intracellular Ca^{++} release seems to be involved. AG has been shown to increase intracellular Ca^{++} concentrations in rat islets, stimulating insulin secretion in a glucose dependent manner (Date et al., 2002). This action may occur via activation of GHS R1a, as stimulation of insulin secretion by AG in INS-1E cells occurs in a Ca^{++} dependent manner and this action is blocked using GHS R1a antagonist [D-Lys3] GHRP-6 (Gauna et al., 2006). Interestingly, this clonal cell line expresses GHS R1a (Colombo et al., 2003), however lacks specific immunoreactivity for GHS R1a (Gauna et al., 2006), questioning if AG and [D-Lys3] GHRP-6 are interacting with GHS R1a or an unknown receptor.

1.7.4 PGDP Regulation of Insulin Secretion is Dose and Glucose Dependent

It appears that the relationship between AG and insulin secretion is complex and is regulated by dosage and glucose concentration. There are reports that only low doses of AG, from 0.01 to 1 nM, are capable of inhibiting insulin secretion under stimulatory concentrations of glucose (8.3, 11.1, 22.2 mM glucose) in mice islets (Reimer, Pacini and Ahren, 2003). In contrast, in the clonal rat beta cell line, INS-1E, the inhibitory action of AG on insulin secretion has been reported to occur at stimulatory glucose concentrations (16.7 and 25mM glucose) but only at a threshold of 0.1 nM ghrelin with a maximal inhibitory response at 1 μ M ghrelin (Colombo et al., 2003). AG has also been shown to affect insulin secretion only at sub and supra physiological concentrations, with 1-100

pM ghrelin inhibiting insulin secretion from mice islets, and 0.1-1 μ M ghrelin stimulating insulin secretion (Salehi et al., 2004, Qader et al., 2008). Furthermore, a stimulatory action of AG on insulin secretion was shown to elicit a greater response from the islets of diabetic rats as compared to control rats (Adegate and Ponery, 2002). It is clear that the conditions that favour inhibition and stimulation of insulin secretion by AG require further exploration.

1.8 PROGHRELIN DERIVED PEPTIDES MODULATE BETA CELL MASS

PGDP not only regulate insulin secretion from pancreatic beta cells, but enhance beta cell proliferation and survival (Granata et al., 2007, Granata et al., 2008, Zhang et al., 2007, Wang et al., 2010b, Wang et al., 2010a, Irako et al., 2006). The ability to positively influence beta cell mass with PGDP would prove desirable in the treatment of T2D, which is associated with a progressive decline in beta cell mass (Dickson and Rhodes, 2004, Lingohr, Buettner and Rhodes, 2002, Weir and Bonner-Weir, 2004, Choi and Woo, 2010, Bernard-Kargar and Ktorza, 2001).

1.8.1 Beta Cell Mass Fluctuates With Insulin Demand

The beta cells in the pancreatic islets show a great capacity for plasticity; beta cell mass varies throughout development (Dickson and Rhodes, 2004, Bernard-Kargar and Ktorza, 2001, Ackermann and Gannon, 2007, Bouwens and Rooman, 2005), and beta cells are further capable of adapting to insulin demand by altering their functional capacity and mass within the endocrine pancreas (Dickson and Rhodes, 2004, Lingohr, Buettner and Rhodes, 2002, Bernard-Kargar and Ktorza, 2001, Bouwens and Rooman, 2005, Kargar and Ktorza, 2008, Pick et al., 1998). Beta cell mass is determined by the

combined effects of beta cell size, proliferation, neogenesis and beta cell death (Rhodes, 2005). During development, beta cell remodeling plays an important role in the establishment of pancreatic mass in the adult organism, with postnatal periods of beta cell apoptosis (Scaglia et al., 1997), followed by cellular proliferation and growth (Montanya et al., 2000). Adult rodents and humans sustain low rates of proliferation to maintain their beta cell mass (Montanya et al., 2000, Dor et al., 2004, Meier et al., 2008). However, beta cell mass can be up-regulated in adult organisms in response to physiological challenges such as the increased insulin demand seen during pregnancy or obesity in humans and rodents (Bernard-Kargar and Ktorza, 2001, Parsons, Brelje and Sorenson, 1992, Muharram et al., 2005, Weir et al., 2001, Jetton et al., 2005). During pregnancy, beta cell mass is enhanced via an increase in proliferation, triggered by placental lactogens (Bernard-Kargar and Ktorza, 2001, Parsons, Brelje and Sorenson, 1992). Beta cell mass is also up-regulated in response to the increased insulin resistance that is associated with obesity (Lingohr, Buettner and Rhodes, 2002, Bernard-Kargar and Ktorza, 2001, Cai et al., 2008).

1.8.2 Beta Cell Mass and Type 2 Diabetes

The regulation of beta cell mass is of great importance to the development of T2D. The progression from obese normoglycemia to T2D is proposed to involve a sequential failure of beta cell function along with a decline in beta cell mass. These individuals are no longer able to compensate for their obesity-induced peripheral insulin resistance and proceed to develop hyperglycemia and T2D (Dickson and Rhodes, 2004, Lingohr, Buettner and Rhodes, 2002, Weir and Bonner-Weir, 2004, Choi and Woo, 2010, Bernard-Kargar and Ktorza, 2001). A decline in beta cell mass has been reported in many

animal models of T2D (Pick et al., 1998, Movassat et al., 1997, Sone and Kagawa, 2005), as well as from clinical studies of T2D in humans (Yoon et al., 2003, Butler et al., 2003).

1.8.3 Akt Signaling is an Important Pathway in the Maintenance of Beta Cell Mass

Akt is a serine-threonine kinase that is implicated as a major regulator of beta cell proliferation and survival (Dickson and Rhodes, 2004, Elghazi, Balcazar and Bernal-Mizrachi, 2006, Elghazi and Bernal-Mizrachi, 2009, Fatrai et al., 2006). Akt is activated by phosphorylation by phosphoinositide dependent kinase-1 (PDK1) (Dickson and Rhodes, 2004, Elghazi and Bernal-Mizrachi, 2009) and mammalian target of rapamycin complex 2 (mTORC2) (Sarbasov et al., 2005), after being recruited to the plasma membrane by binding to phosphatidylinositol (3,4,5) P3 phosphate (PIP3) (produced by PI3K signaling) (Dickson and Rhodes, 2004, Ackermann and Gannon, 2007). Activated Akt then proceeds to activate and inhibit a diverse range of signaling pathways leading to increased cellular proliferation, as well as inhibition of apoptosis (Dickson and Rhodes, 2004, Fatrai et al., 2006, Elghazi et al., 2007, Franke, 2008, Franke et al., 2003). Akt enhances proliferation by activation of the cell cycle regulator, cyclin-dependent kinase 4 (cdk4) via mammalian target of rapamycin complex 1 (mTORC1) signaling (Fatrai et al., 2006, Balcazar et al., 2009, Rachdi et al., 2008) and inhibition of signaling molecules with negative effects on beta cell mass such as glycogen synthase kinase 3 (GSK3) (Mussmann et al., 2007) and the transcription factor forkhead box O1 (FoxO1) (Kops et al., 1999, Glauser and Schlegel, 2007, Martinez et al., 2006). Akt also modulates survival by inhibiting apoptosis by inactivating the pro-apoptotic protein BAD and caspase 9 (Elghazi and Bernal-Mizrachi, 2009, Franke et al., 2003).

The effects of Akt on cell proliferation and survival is evident in transgenic mice with a constitutively active pancreatic Akt. These mice have a dramatic enhancement in their beta cell mass, including increased number of proliferating beta cells per islet and increased cell size (Bernal-Mizrachi et al., 2001, Tuttle et al., 2001). Furthermore, these mice are resistant to developing diabetes in response to streptozotocin, suggesting that the constitutive signaling through Akt is protective (Bernal-Mizrachi et al., 2001, Tuttle et al., 2001). As well, non-diabetic Zucker fatty (*fa/fa*) rats are insulin resistant, but have a nearly 4 fold increase in beta cell mass and high levels of beta cells immunopositive for phosphorylated Akt (Jetton et al., 2005). Akt is also involved in beta cell function as seen in transgenic mice expressing a dominant negative Akt in their beta cells. Partial blockade of endogenous Akt signaling in these mice creates a deficit in insulin secretion and a propensity towards the development of T2D when fed HFD (Bernal-Mizrachi et al., 2004).

Glucose, insulin-like growth factor 1 (IGF-1), insulin and the incretins, GLP-1 and GIP, have all been shown to elicit positive effects on beta cell proliferation and survival by activation of Akt signaling (Dickson and Rhodes, 2004, Lingohr, Buettner and Rhodes, 2002, Elghazi, Balcazar and Bernal-Mizrachi, 2006, Li et al., 2005, Wang et al., 2004, Wang and Brubaker, 2002, Navarro-Tableros et al., 2004, Srinivasan et al., 2002, Hui et al., 2003, Liu et al., 2002, Ohsugi et al., 2005, Hoorens et al., 1996).

1.8.4 Apoptosis and Beta Cell Mass

Regulation of apoptosis is an important factor in the maintenance of beta cell mass (Choi and Woo, 2010, Pick et al., 1998, Sone and Kagawa, 2005). Programmed cell death, or apoptosis, can be triggered by external (via cell death receptor) or internal

signals (mitochondrial or endoplasmic reticulum dysfunction) (Morishima et al., 2002, Li et al., 1997, Budihardjo et al., 1999). Caspase 3 is one of the main effectors of the apoptotic induction pathway, inactivating DNA repair enzymes and adhesion molecules, while disregulating the integrity of the cytoskeleton (Nicholson et al., 1995, Wen et al., 1997, Kothakota et al., 1997, Porter and Janicke, 1999, Fernandes-Alnemri, Litwack and Alnemri, 1994). Of particular relevance to the beta cell, is apoptosis initiated by mitochondrial dysfunction given the absolute importance of this organelle in the coupling of glucose sensing to insulin secretion (Szabadkai and Duchon, 2009). Mitochondrial dysfunction results in increased mitochondrial membrane permeability, leading to the release of cytochrome c from the mitochondrion; once in the cytosol, cytochrome c forms a complex with apoptotic protease activating factor 1 (Apaf-1) and procaspase 9, the latter of which becomes proteolytically cleaved to form caspase 9. Activated caspase 9 is then able to cleave and activate procaspase 3 (Budihardjo et al., 1999). Apoptotic cells display a specific phenotype, including condensation of chromatin, DNA fragmentation, and formation of apoptotic bodies (Kerr, Wyllie and Currie, 1972). Activated caspase 3 is responsible for the morphological changes occurring during apoptosis, including nuclear condensation and fragmentation of DNA (Wen et al., 1997, Kothakota et al., 1997, Porter and Janicke, 1999, Janicke et al., 1998, Widlak, 2000).

There are various methods of inducing apoptosis in cells and tissues, including serum/growth factor starvation, cytokine treatment, glucotoxicity, lipotoxicity as well as a number of synthetic compounds. Particularly relevant to beta cells and T2D models are glucotoxicity and lipotoxicity. Models of lipotoxicity, such as prolonged incubation with a high concentration of the free fatty acids oleate or palmitate, strongly induce apoptosis

in beta cells (Lai et al., 2008, Koshkin et al., 2008, Wrede et al., 2002, Maestre et al., 2003).

1.8.5 PGDP Regulate Beta Cell Mass

The potential protective effect of PGDP on beta cells could prove promising for treatment of T2D, as PGDP have been reported to increase in beta cell mass. AG, UAG and obestatin have been reported to increase beta cell survival both *in vitro* and *in vivo* including in preparations of human islets (Granata et al., 2007, Irako et al., 2006). AG (Granata et al., 2007, Zhang et al., 2007), UAG (Granata et al., 2007) and obestatin (Granata et al., 2008) increase cell proliferation and have an anti-apoptotic effect in both beta cell lines and rodent islets via the mitogen activated protein kinase (MAPK) and PI3K/ Akt pathways. Furthermore, ghrelin treatment following streptozotocin injection in neonatal rats increases the amount of proliferating beta cells and prevents the appearance of hyperglycemia (Irako et al., 2006). Similar restorative effects have also been reported with ghrelin treatment following pancreatectomy (Kerem et al., 2008).

The pro-proliferative and pro-survival effect of PGDP on beta cells may occur via paracrine signaling in the pancreas. The beta cell line HIT-T15 expresses preproghrelin mRNA and secretes both AG and UAG (Granata et al., 2007). Adding anti-ghrelin antibody to HIT-T15 culture increases apoptosis, suggesting that blockade of the ghrelin secreted from these cells may be impairing survival (Granata et al., 2007). Similar effects are achieved with the addition of anti-obestatin antibody to HIT-T15 cells, indicating that endogenous pancreatic obestatin may also play a local role in the maintenance of beta cell survival (Granata et al., 2008).

1.9 STATEMENT OF OBJECTIVES

The presence of a developmentally sensitive population of ghrelin cells within pancreatic islets (Chanoine and Wong, 2004, Nakahara et al., 2006), along with the modulatory influence of PGDP on insulin secretion (Lee et al., 2002, Granata et al., 2007, Wierup et al., 2004, Gauna et al., 2006, Granata et al., 2008, Dezaki et al., 2006, Esler et al., 2007, Date et al., 2002, Adeghate and Ponery, 2002, Salehi et al., 2004, Qader et al., 2008, Reimer, Pacini and Ahren, 2003, Qader et al., 2005, Dezaki et al., 2004, Dezaki, Kakei and Yada, 2007, Colombo et al., 2003, Wang et al., 2010c, Egido et al., 2009, Ren et al., 2008) and beta cell mass (Granata et al., 2007, Granata et al., 2008, Zhang et al., 2007, Wang et al., 2010b, Wang et al., 2010a, Irako et al., 2006) suggests that ghrelin plays an important regulatory role in the endocrine pancreas. The ability of AG to affect insulin secretion in the presence and absence of the traditional ghrelin receptor, GHS R1a, illustrates that the actions of AG on beta cells are complex and our understanding is far from complete. The actions of UAG and obestatin in beta cell function are also mostly unknown. Despite the many unknown elements of pancreatic PGDP actions, it remains clear that given the potential to modulate insulin secretion and beta cell proliferation and survival, manipulation of PGDP in the endocrine pancreas may enable us to influence two important hallmarks of T2D, beta cell function and mass.

The purpose of the current work was to characterize the actions of PGDP on the maintenance of beta cell mass and the regulation of insulin release. We have utilized two rodent pancreatic beta cell models, INS-1 and MIN6 cells (rat and mouse insulinoma cell lines, respectively) to explore the actions of PGDP on beta cell proliferation and survival. We have found MIN6 cells express GHS R1a, while we have shown that INS-1 cells lack

GHS R1a. Using these cells we were able to investigate the necessity of GHS R1a for the actions of PGDP in beta cell proliferation and survival. Furthermore, we investigated PGDP regulation of insulin secretion in INS-1 cells to identify if PGDP regulation of insulin secretion occurs independently of GHS R1a. Insulin secretion was determined over a physiological dose range of AG, UAG and obestatin and a wide range of glucose backgrounds to determine if the affect of PGDP is dose or glucose dependent.

CHAPTER 2 MATERIALS AND METHODS

2.1 CELL CULTURE AND REAGENTS

The rat insulinoma cell line, INS-1 was kindly provided by Dr. Wang (St. Michael's Hospital, Ontario). INS-1 cells were maintained with RPMI 1640 media (11mM glucose) (Invitrogen, Burlington, Ontario) supplemented with 12% fetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, 100 U/ml penicillin and 100 μ g/ml streptomycin. The mouse insulinoma cell line, MIN6 was kindly provided by Dr. Mbikay (Ottawa Health Research Institute, Ontario). MIN6 cells were maintained with DMEM (4500 mg glucose/L, L-glutamine, sodium bicarbonate and pyridoxine.HCl) (Sigma-Alrich, St. Louis MO, USA) supplemented with 15% FBS, 50 μ M 2-mercaptoethanol, 100U/ml penicillin and 100 μ g/ml streptomycin. The mouse glucagon secreting cell line, Alpha-TC1 was kindly provided by Dr. Brubaker (University of Toronto, Ontario). Alpha-TC1 cells were maintained with DMEM (4500 mg glucose/L, L-glutamine, sodium bicarbonate and pyridoxine.HCl) (Sigma-Alrich, St. Louis MO, USA) supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin. Human medullary thyroid carcinoma cell line, TT were purchased from American Type Culture Collection (VA, USA) and maintained in Ham's F12 media (L-glutamine, sodium bicarbonate) (Invitrogen, Burlington, Ontario) supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were incubated at 37°C at an atmosphere of 95 % air and 5% CO₂.

Peptides (rat AG, UAG and obestatin) were obtained from Bachem (PA, USA). Unless other wise stated, all chemicals were purchased from Sigma-Alrich (St. Louis MO, USA).

2.2 GHS R 1A DETECTION

2.2.1 rt - PCR

RNA was extracted from mouse tissues (hypothalamus and pancreas) using Trizol (Invitrogen, USA) (1 ml/100mg tissue). Tissues were homogenized, RNA was precipitated using isopropanol, and RNA pellets were resuspended in diethylpyrocarbonate (DEPC) (Sigma-Alrich, St. Louis MO, USA) treated water. RNA concentration was determined by absorbance readings at 260 nm with a spectrophotometer. The formula used to calculate RNA concentration was $[\text{RNA sample}] = 40 \times A_{260} \times \text{dilution factor}$, where 40 represents the $\mu\text{g/ml}$ of RNA with an absorbance of 1 unit at 260 nm. RNA was extracted from INS-1, MIN6, alpha-TC, and TT cells with an RNeasy Kit (Qiagen, Mississauga, Ontario). The concentration of RNA from both tissues and cell lines were determined by spectrophotometry and 6 μg RNA was DNaseI treated (Invitrogen, Burlington, Ontario) according to manufacturers instructions. Two μg of DNaseI-treated RNA was primed with random hexamers, and treated with reverse transcriptase to produce cDNA according to manufacturer's instructions (SuperScript II Reverse Transcriptase, Invitrogen, Burlington, Ontario). cDNA was stored at -20°C until further use. PCR reactions were performed using BioRad supermix (Biorad, Mississauga, Ontario) in a volume of 25 μl , according to manufacturer's instructions. Primers were purchased from IDT (CA, USA); sequences are as follows: mouse GHS R1a (mGHS R1a) forward 5'-CTATCCAGCATGGCCTTCTC-3' and reverse 5'-GGAAGCAATGGCGAAGTAG-3'; human GHS R1a (hGHS R1a) forward 5'-CTGCGCTCAGGGACCAGAACCA -3' and reverse 5' -GTTGATGGCAGCACTGAGGTAGAA -3'; rat GHS R1a (rGHS R1a) forward 5'-

CGACCTGCTCTGCAAACCTC-3' and reverse 5'-CACGCCACACCAGCACGAAGA-3';
18s forward 5'-TCAACTTTCGATG GTAGTCGCCGT-3' and reverse 5'-
TCCTTGGATGTGGTAGCCGTTTCT-3'. The mouse primers produce an amplicon of
198 bp, and hybridize to the mouse GHS R1a mRNA sequence as follows: forward
primer hybridizes at 491 bp and reverse primer hybridizes at 689bp. The human primers
produce an amplicon of 205 bp and hybridize to the human GHS R1a mRNA sequence as
follows: forward primer hybridizes at 795 bp and reverse primer hybridizes at 1000 bp.
The rat primers produce an amplicon of 216 bp and hybridize to the rat GHS R1a mRNA
sequence as follows: forward primer hybridizes at 333 bp and reverse primer hybridizes
at 549 bp. Due to sequence similarity all of the primer sets were indicated to amplify
products from mouse, rat and human samples. Amplification cycles were carried out as
follows: 10 minutes at 95°C, then 40 cycles of 95°C for 30 seconds, 60°C for 1 minute,
72°C for 30 seconds. PCR products were analyzed on 1% agarose gels and then
visualized with ethidium bromide. The identity of all PCR products was confirmed by
DNA sequencing.

2.2.2 Western Blot

Total protein collected from TT, MIN6 and INS-1 cells was homogenized in
cytobuster (Novagen, USA) with protease and phosphatase inhibitor tablets (Roche,
Laval, Quebec) and centrifuged at 14 000 rpm before quantification with Bradford
protein assay reagents (Biorad, Mississauga, Ontario). Briefly, 40µg of protein was
separated by 10% SDS-PAGE and transferred onto PVDF membrane (Biorad,
Mississauga, Ontario). Blots were probed with the appropriate primary and secondary

antibodies (see below for details) in 5% milk blocking solution. Blots containing TT, MIN6 and INS-1 untreated cell extracts were incubated with rabbit anti-rat GHS R1a antibody (Alpha Diagnostics, TX, USA) (5 µg/ml). Quality of loaded protein was confirmed by reprobng the blots (Re-Blot plus (mild solution), Millipore, CA, USA) for actin using actin rabbit polyclonal primary antibody (1:500) (Sigma-Aldrich, St Louis, MO USA). GHS R1a and actin immunoreactive bands were visualized with donkey anti-rabbit IgG (1:10000) (GE Healthcare UK Limited, Quebec) secondary antibody. Bands were visualized with Western C detection reagents (Biorad, Mississauga, Ontario). Images were captured with the ChemiDoc XRS imaging system (Biorad, Mississauga, Ontario).

2.3 PALMITATE PREPARATION

Palmitate stock solution (100mM) was prepared in 0.1M NaOH and heated at 70°C. Palmitate was diluted to a concentration of 5mM in 5% free fatty acid (FFA) - free BSA (weight/volume in ddH₂O) and complexed by heating at 60°C. Further dilutions for treatments were completed using the appropriate serum free media. Treatments were filter sterilized with 0.2µm filters before use.

2.4 PROLIFERATION

2.4.1 Cell Counts

Cells were plated in 6 well plates and were grown under normal conditions until reaching 50% confluency. Cells were then treated for 24 hours in serum free media with or without peptide hormone treatments (AG, UAG and obestatin at concentrations of 10⁻¹³, 10⁻¹¹, 10⁻⁹ and 10⁻⁷M) at 37°C at an atmosphere of 95 % air and 5% CO₂. At the

end of the incubation period, wells were rinsed in 1X PBS and cells were removed from the plates with trypsin/EDTA. Cells in suspension were pelleted by centrifugation at 2500 rpm for 5 minutes and the resulting pellets were resuspended in equal volumes of PBS. Cell number (above a threshold size of 10 μm) and mean cell size were determined with a Beckman Coulter particle counter.

2.4.2 BrdU Assay

BrdU (5-bromo-2'-deoxyuridine) is a pyrimidine analog, and as such is incorporated in the DNA during mitosis in place of thymidine. Addition of this compound to proliferating cells results in the incorporation of a detectable molecule that can be measured to indicate the levels of proliferation at the time of treatment. We utilized a BrdU kit (Roche, Laval, Quebec) that detects the amount of BrdU incorporated into cell culture via an anti-BrdU antibody linked to a peroxidase. Subsequent application of tetramethyl-benzidine, a substrate for peroxidase, results in a product which is quantifiable after measurement of the absorbance at 370nm.

MIN6 and INS-1 cells were plated at a density of 100 000 cells/well in 96 well plates. Assays were performed the following day. Two experimental protocols were followed: 1) serum starvation - cells were incubated in serum free media with or without peptide treatment (AG, UAG or obestatin at 10^{-7}M) for 24 hours, 2) FFA treatment (0.2 mM palmitate) - cells were pretreated overnight in serum free media with or without peptide treatment (AG, UAG or obestatin at 10^{-7}M) before a 24 hour incubation with 0.2 mM palmitate with or without peptide treatment (continuation of pretreatment: AG, UAG or obestatin at 10^{-7}M). BrdU labeling solution was added to culture (final concentration of 10 μM) at the beginning of the treatment period. At the completion on the treatment

period, all media was aspirated and plates were stored at 4°C overnight as per kit protocol. The following day the cells were treated with the supplied fixative/denaturing agent for 30 minutes at room temperature. Anti-BrdU antibody linked to peroxidase was applied to cells for 90 minutes at room temperature. Plates were then rinsed and the substrate solution was applied to wells. The absorbance of the reaction was measured at 370 nm (background 492 nm) and readings were obtained 30 minutes into the reaction.

2.5 APOPTOSIS

2.5.1 Hoechst Nuclear Staining

MIN6 and INS-1 cells were plated in chamber slides and grown until 80% confluency. Cells were incubated overnight at 37°C and an atmosphere of 95 % air and 5% CO₂ in serum, serum free media supplemented with 0.5% FFA free BSA, or 0.2 mM palmitate (prepared as described above) with or without AG, UAG or obestatin (10⁻⁷M). At the end of the 24 hour incubation, hoechst nuclear dye 33342 was added to the wells at a concentration of 10 µg/ml for a further 15 minutes at 37°C. Cells were then rinsed with 1X PBS, and fixed in 4% paraformaldehyde (PFA) in PBS at room temperature for 15 minutes. Slides were then coverslipped with mounting media. Slides were visualized by fluorescent microscopy. An experimenter blind to treatment condition performed analysis of apoptotic cells using condensation of the nucleus and the presence of apoptotic bodies as positive markers of apoptotic cells, as these are phenotypic indicators of late stage apoptosis (Kerr, Wyllie and Currie, 1972).

2.5.2 Caspase 3 Assay

Caspase 3 is an enzyme that is responsible for the proteolytic cleavage of many intracellular proteins during the apoptotic process leading towards cell death (Porter and Janicke, 1999). However, prior to activation, caspase 3 must be cleaved by caspase 9 (Li et al., 1997). The concentration of activated caspase 3 can be quantified by incubation with a substrate that upon cleavage results in a product is measurable by absorbance.

Cleaved caspase 3 levels were measured with a colorimetric caspase 3 assay kit (Sigma-Aldrich, St. Louis MO, USA). Cells were plated on 6 well plates and grown to 80% confluence before apoptotic induction. Apoptosis was achieved through two separate protocols: 1) 48 hour serum deprivation and 2) lipotoxicity via 24 hour palmitate treatment. Treatments were performed in the absence and presence of AG, UAG or obestatin (serum deprivation protocol: peptide dose range (10^{-13} , 10^{-11} , 10^{-9} and 10^{-7} M), palmitate treatment: peptides all 10^{-7} M). Following treatment, cells were lysed in the kit lysis buffer, incubated on ice for 20 minutes and then centrifuged at 16000rpm, for 15 minutes, at 4°C. The resulting supernatant was stored at -80°C until later use. Thirty μ l of the supernatant was incubated in the presence of the caspase 3 substrate, acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA). Caspase 3 cleavage of this substrate results in release of the product p-nitroaniline, the concentration of which was then quantified at 405nm.

2.6 AKT SIGNALING

Akt is activated by phosphorylation, thus we measured the amount of phosphorylated Akt as an indicator of Akt signaling (Dickson and Rhodes, 2004, Elghazi and Bernal-Mizrachi, 2009). Prior to protein extraction, INS-1 and MIN6 cells were pre-

incubated in serum free culture media for 4 hours before a 10 minute treatment in serum free media with or without peptide treatment (AG, UAG or obestatin (10^{-11} , 10^{-9} and 10^{-7} M)). Akt phosphorylation was quantified by western blot band density analysis. Blots were probed with specific antibodies (pAkt rabbit polyclonal IgG (1:500) (Santa Cruz Biotechnology, CA, USA), Akt mouse polyclonal IgG (1:500) (Santa Cruz Biotechnology, CA, USA), actin rabbit polyclonal (1:500) (Sigma-Aldrich, St Louis, MO USA). Immunoreactive bands were visualized with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (1:10000) (Santa Cruz Biotechnology CA, USA) or donkey anti-rabbit IgG (1:10000) (GE Healthcare UK Limited, Quebec). Band density was quantified using Quantity One software (Biorad, Mississauga, Ontario). Band density was evaluated and was reported as the ratio of pAkt/Akt.

2.7 INSULIN SECRETION

INS-1 cells were plated at a density of 200 000 cells/well in 24 well plates. Cells were maintained as described above until reaching ~ 80 % confluence. Cells were rinsed twice with PBS before a 1 hour incubation in glucose free KRBH (140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4 , 0.5 mM MgSO_4 , 1.5 mM CaCl_2 , 2 mM NaHCO_3 , 10 mM HEPES (pH7.4) and 0.1% BSA). Peptide treatments were prepared in KRBH of varying glucose concentrations (2, 5, 11, 16 and 25 mM glucose). Cells were incubated with or without the peptide treatments (10^{-13} , 10^{-11} , 10^{-9} and 10^{-7} M) for 1 hour after which media was immediately collected and stored at -20°C until further use. Insulin concentration was measured by radioimmunoassay (RIA) (Rat Insulin RIA kit, Linco Research (MO, USA)). Total protein was collected from the plates using cytobuster (Novagen, USA). Protein was sonicated and centrifuged at 14 000 rpm before quantification with Bradford

protein assay (Biorad, Mississauga, Ontario). Insulin concentration was normalized to total protein content of the wells (ng insulin/ μg protein).

2.8 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Samples for cell counting, serum starvation caspase 3 assays, Akt phosphorylation, and insulin secretion experiments were performed in triplicate and experiments were repeated once. Samples for the palmitate proliferation experiments were performed in groups of 8. Samples for the nuclear staining experiments were performed in duplicate with counts obtained from 3 views of each slide. The samples for palmitate caspase 3 assays were performed in triplicate.

Data were analyzed using GraphPad Prism software (v4.0). Data are reported as mean \pm SEM. One way ANOVAs were used for multiple comparisons, followed by Dunnett's Multiple Comparison test for post hoc analysis of experimental peptide vs. control treatment. Differences between groups with a p value < 0.05 were considered significant.

CHAPTER 3 RESULTS

3.1 GHS R1A IS PRESENT IN MIN6 CELLS, BUT NOT INS-1 CELLS

Both MIN6 and INS-1 pancreatic beta cell lines were tested for the presence of GHS R1a transcript and protein. Mouse hypothalamus and pancreas served as a positive control for GHS R1a, as these tissues have been reported to contain GHS R1a. Indeed, we have confirmed the presence of the GHS R1a in mouse hypothalamus and pancreas using the primer set based on the mouse GHS R1a sequence (Figure 3a). MIN6 and INS-1 cell lines were tested for GHS R1a expression using 3 independent primer sets (1. mGHS R1a based on the mouse sequence of GHS R1a, 2. hGHS R1a based on the human sequence of GHS R1a and 3. rGHS R1a based on the rat sequence of GHS R1a. Given the conservation of the GHS R1a sequence across these species, all three of the independent primer sets were indicated to amplify GHS R1a transcript against the mouse, rat and human GHS R1a sequences. The human medullary thyroid cell line, TT, was also included in our analysis as a positive control, as we have shown TT cells to express GHS R1a (unpublished data). The mouse glucagon secreting cell line, alpha-TC was tested for GHS R1a expression as well, to investigate the presence of GHS R1a in other islet cell types. As expected, TT cells expressed GHS R1a (Figure 3b), which was detectable using all three primer sets, indicating a degree of similarity in the GHS R1a transcript across rodents to humans. MIN6 also contained the GHS R1a transcript, which like TT cells, was detectable using all three primers (Figure 3c). Using two independent samples of INS-1 cDNA, we were unable to detect GHS R1a transcript using any of the three primer sets (Figure 3d), indicating that INS-1 cells lack GHS R1a expression. GHS R1a was

Figure 3. MIN6, but not INS-1 cells express GHS R1a. MIN6 and INS-1 cells were probed for the presence of GHS R1a transcript and protein. rt PCR reactions were performed on cDNA made from mouse tissues or TT, MIN6, INS-1 and alpha-TC cells. PCR products were separated on 1% agarose gels and visualized with ethidium bromide. Samples were probed for 18s to check for sample quality. A) Mouse hypothalamus and pancreas express GHS R1a. cDNA from mouse hypothalamus and pancreas was used as a positive control for GHS R1a expression using GHS R1a primers based on mouse GHS R1a. B) The human medullary thyroid cell line, TT cells, was used as a positive control for GHS R1a in our analysis of cell lines. Three independent primer sets for GHS R1a expression were tested (1. mGHS R1a based on the mouse sequence of GHS R1a, 2. hGHS R1a based on the human sequence of GHS R1a, and 3. rGHS R1a based on the rat sequence of GHS R1a). C) GHS R1a expression is detectable in MIN6 cells using all three independent GHS R1a primer sets. D) GHS R1a expression was not detectable in two independent INS-1 cDNA samples, probed with 3 independent GHS R1a primer sets. However, GHS R1a expression was detectable in alpha-TC cells using all three independent GHS R1a primer sets. E) Lysates from TT, MIN6 and INS-1 cells were analyzed by western blotting for the presence of GHS R1a protein. TT cell lysate produced a weak 27 kDa band representing GHS R1a protein. This 27 kDa band was pronounced in MIN6 lysate but absent from INS-1 cell lysate, confirming the rt PCR results.

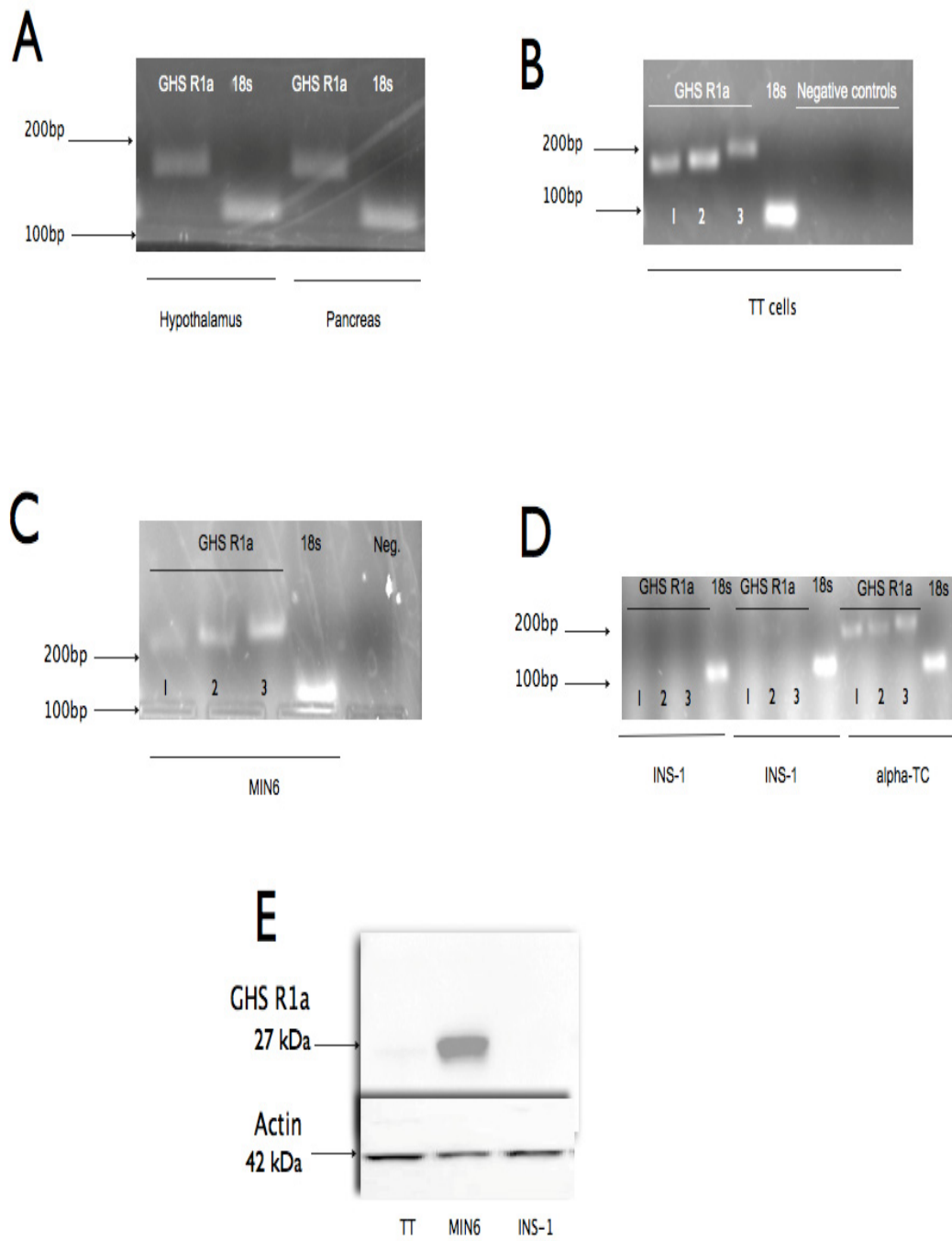


Figure 3

detectable in alpha-TC cells (Figure 3d). Our finding that MIN6 but not INS-1 cells contain GHS R1a, was confirmed by western blotting of cellular protein lysates using a rabbit anti-rat GHS R1a antibody (Alpha Diagnostics, TX, USA), which showed a robust band from MIN6 lysate, and no detectable band in INS-1 cell lysate (Figure 3e). These results indicate that MIN6 have GHS R1a, while INS-1 lack this traditional receptor for AG.

3.2 BOTH ACYLATED GHRELIN AND UNACYLATED GHRELIN INCREASE/PRESERVE CELL NUMBER IN MIN6 AND INS-1 CELLS

Both average cell size and cell number was determined in MIN6 and INS-1 cells in response to serum starvation in the absence and presence of PGDP. Average cell size was used as a measure of cellular hypertrophy, while cell number was used as a preliminary measure of cell proliferation and/or apoptosis. MIN6 and INS-1 cells were treated with serum free media with or without AG, UAG or obestatin for 24 hours. PGDP did not alter average cell size in either INS-1 (Figure 4a-c) or MIN6 cells (Figure 5a-c). In INS-1 cells, AG at all doses tested (10^{-13} M, 10^{-11} M, 10^{-9} and 10^{-7} M) increased cell number relative to serum starved control cells (Figure 4d) (10^{-13} M AG: $1.28 \times 10^6 \pm 0.07 \times 10^6$ cells ($p < 0.05$), 10^{-11} M AG: $1.38 \times 10^6 \pm 0.11 \times 10^6$ cells ($p < 0.01$), 10^{-9} M AG: $1.47 \times 10^6 \pm 0.07 \times 10^6$ cells ($p < 0.01$) and 10^{-7} M AG: $1.16 \times 10^6 \pm 0.08 \times 10^6$ cells ($p < 0.05$), vs. serum starved controls: $0.63 \times 10^6 \pm 1.7 \times 10^6$ cells). Similar to AG, UAG at all doses tested (10^{-13} M, 10^{-11} M, 10^{-9} and 10^{-7} M) increased INS-1 cell number relative to serum starved control cells (Figure 4e) (10^{-13} M UAG: $2.03 \times 10^6 \pm 0.3 \times 10^6$ cells ($p < 0.01$), 10^{-11} M UAG: $2.79 \times 10^6 \pm 0.18 \times 10^6$ cells ($p < 0.001$), 10^{-9} M UAG: $2.18 \times 10^6 \pm 0.11 \times 10^6$ cells ($p < 0.01$) and 10^{-7} M UAG: $3.12 \times 10^6 \pm 0.17 \times 10^6$ cells ($p < 0.001$), vs. serum

Figure 4. Acylated ghrelin (AG) and unacylated ghrelin (UAG) increase cell number in serum starved INS-1 cells. INS-1 cells were plated in 6 well plates and grown to 50% confluency. INS-1 cells were then incubated for 24 hours serum free (SF) media with or without AG, UAG or Obestatin (Ob) (10^{-13} , 10^{-11} , 10^{-9} , 10^{-7} M). Average cell size and cell number was analyzed with a particle counter. Data is expressed as mean \pm SEM. Serum starvation or PDGP treatment did not change average cell size (A-C). Incubation in SF media significantly decreased cell number as compared to cells incubated in media containing FBS (serum) (D-F). AG (D) and UAG (E) (all concentrations) significantly increased cell number compared to SF cells, while Ob had no significant effect on cell number (F). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to SF, analyzed with an ANOVA followed by Dunnet's post hoc test. Samples were performed in triplicate and experiments were completed twice.

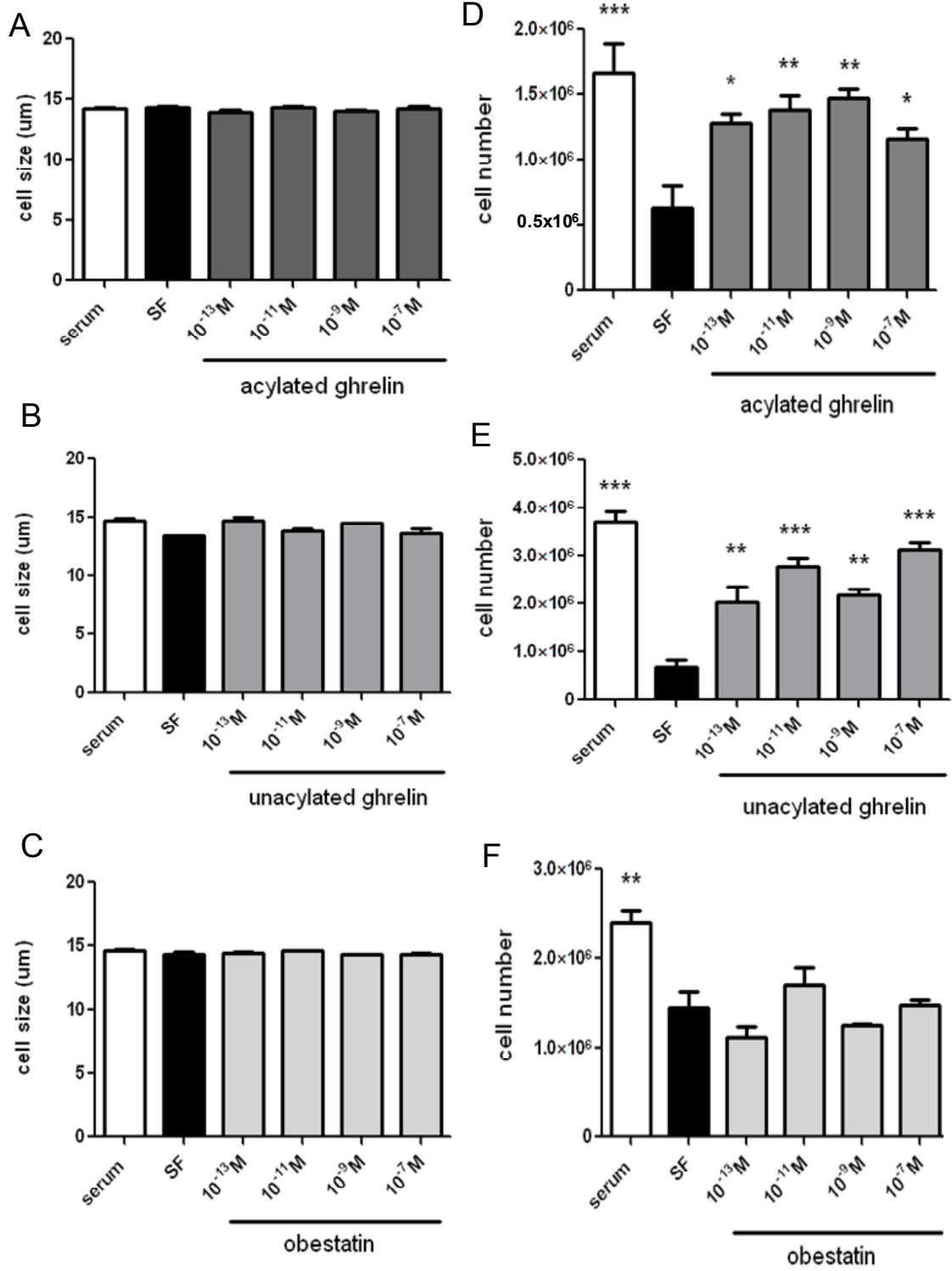


Figure 4

Figure 5. Acylated ghrelin (AG), unacylated ghrelin (UAG) and obestatin (Ob) increase cell number in serum starved MIN6 cells. MIN6 cells were plated in 6 well plates and grown to 50% confluency. MIN6 cells were then incubated for 24 hours serum free (SF) media with or without AG, UAG or Ob (10^{-13} , 10^{-11} , 10^{-9} , 10^{-7} M). Average cell size and cell number was analyzed with a particle counter. Data is expressed as mean \pm SEM. Serum starvation or PDGP treatment did not change average cell size (A-C). Incubation in SF media significantly decreased cell number as compared to cells incubated in media containing FBS (serum) (D-F). AG (all concentrations) significantly increased cell number compared to SF cells (D), while only 10^{-11} and 10^{-7} M UAG (E) and 10^{-9} and 10^{-7} M Ob (F) significantly increased cell number. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to SF, analyzed with an ANOVA followed by Dunnet's post hoc test. Samples were performed in triplicate and experiments were completed twice.

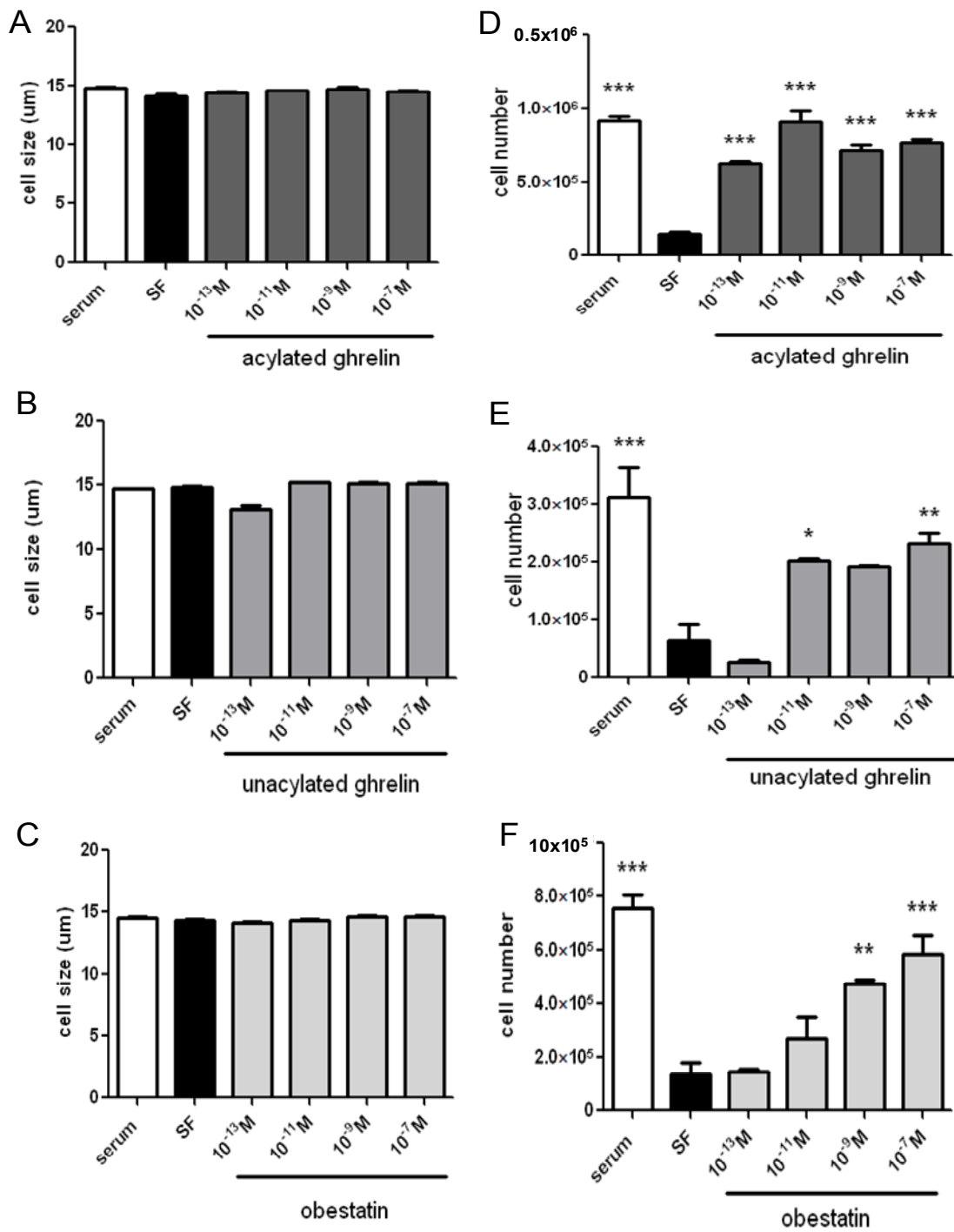


Figure 5

starved controls: $0.69 \times 10^6 \pm 0.13 \times 10^6$ cells). However, obestatin was unable to increase cell number at any dose tested (Figure 4f).

In MIN6 cells, AG at all doses tested (10^{-13} M, 10^{-11} M, 10^{-9} and 10^{-7} M) increased cell number relative to serum starved control cells (Figure 5d) (10^{-13} M AG: $0.62 \times 10^6 \pm 0.16 \times 10^5$ cells ($p < 0.001$), 10^{-11} M AG: $0.91 \times 10^6 \pm 0.76 \times 10^5$ cells ($p < 0.001$), 10^{-9} M AG: $0.71 \times 10^6 \pm 0.37 \times 10^5$ cells ($p < 0.001$) and 10^{-7} M AG: $0.76 \times 10^6 \pm 0.25 \times 10^5$ cells ($p < 0.001$), vs. serum starved controls: $0.14 \times 10^6 \pm 0.12 \times 10^5$ cells). UAG also enhanced cell number relative to serum starved cells, with doses at 10^{-11} M (UAG: $0.2 \times 10^6 \pm 0.47 \times 10^4$ cells ($p < 0.05$)) and 10^{-7} M (UAG: $0.23 \times 10^6 \pm 1.8 \times 10^4$ cells ($p < 0.01$)) resulting in increased cell number (serum starved controls: $0.06 \times 10^6 \pm 0.29 \times 10^5$ cells) (Figure 5e). Obestatin treatment increased cell number in a dose dependent manner with significant responses at doses at 10^{-9} M (Ob: $0.47 \times 10^6 \pm 0.17 \times 10^5$ cells ($p < 0.01$)) and 10^{-7} M (Ob: $0.59 \times 10^6 \pm 0.69 \times 10^5$ cells ($p < 0.001$)) compared with serum starved controls ($0.14 \times 10^6 \pm 0.44 \times 10^5$ cells) (Figure 5f).

We have found that AG and UAG increase cell number in INS-1 cells, while obestatin has no effect on cell number. In contrast, all three PGDP positively regulate cell number in MIN6 cells

3.3 PROGHRELIN DERIVED PEPTIDES INCREASE PROLIFERATION IN INS-1, BUT NOT MIN6 CELLS

To further explore the increase in beta cell number with PGDP we determined if PGDP increase proliferation of INS-1 or MIN6 cells. BrdU incorporation was used as a measure of cellular proliferation. BrdU is a pyrimidine analog and is incorporated in the DNA during mitosis in place of thymidine. Incubation of BrdU with proliferating cells

results in the incorporation of a detectable molecule that can be measured to indicate the levels of proliferation at the time of treatment. Incorporated BrdU was measured via an anti-BrdU antibody linked to a peroxidase. Subsequent application of a peroxidase substrate results in a colour product which is quantifiable after measurement of absorbance at 370nm.

Serum starvation of INS-1 cells resulted in a significant decrease in proliferation (Figure 6a) (OD at 370nm serum starvation condition: 1.58 ± 0.6 compared with serum condition: 2.73 ± 0.08 ($p < 0.05$). AG, UAG and obestatin treatment (10^{-7} M) were all capable of restoring cellular proliferation to that of the serum positive condition (Figure 6a) (OD at 370nm - AG: 3.04 ± 0.04 ($p < 0.01$), UAG: 2.59 ± 0.08 ($p < 0.05$), Ob: 2.62 ± 0.14 ($p < 0.05$). Proliferation of MIN6 cells was slightly, but significantly reduced by serum starvation (Figure 6c) (OD at 370nm serum condition: 3.63 ± 0.23 compared with serum starvation: 3.17 ± 0.07 ($p < 0.05$)). However, neither AG, UAG or obestatin were capable of restoring cell proliferation (Figure 6c).

Palmitate significantly reduced proliferation of INS-1 cells (Figure 6b) (OD at 370nm Palmitate: 1.59 ± 0.13 compared with both serum: 2.898 ± 0.039 and serum free media with 0.5% BSA: 2.287 ± 0.018 ($p < 0.001$)). However, PGDP were unable to restore proliferation under this model of lipotoxicity (Figure 6b). Palmitate produced a non significant reduction in proliferation of MIN6 cells (Figure 6d) (OD at 370nm Palmitate: 1.68 ± 0.14 compared with both serum: 2.10 ± 0.062 and serum free media with 0.5% BSA: 2.052 ± 0.22 (non significant)) and PGDP were without further effect (Figure 6d).

Figure 6. Acylated ghrelin (AG), unacylated ghrelin (UAG) and obestatin (Ob) increase proliferation in serum starved INS-1 cells. INS-1 and MIN6 cells were seeded at a density of 100 000 cells/ well in 96 well plates and grown overnight. Cells were then incubated 1) for 24 hours in serum free (SF) media with or without AG, UAG or Ob (10^{-7} M) or 2) pretreated with AG, UAG or obestatin (10^{-7} M) overnight before a 24 hour treatment with 0.2mM palmitate with or without continued treatment with AG, UAG or Obestatin (Ob) (10^{-7} M). Treatments occurred concurrently with a 24 hour BrdU incorporation period. Cellular proliferation was analyzed with a BrdU kit (Roche, Laval, Quebec). Data is expressed as mean \pm SEM. A) Incubation in SF media significantly decreased cell proliferation in INS-1 cells as compared to cells incubated in media containing FBS (serum). AG, UAG and Ob restored proliferation to that of serum treated control cells. B) Palmitate (0.2mM) significantly decreased proliferation in INS-1 cells as compared to serum and 0.5% BSA controls. Pretreatment with PGDP was unable to restore proliferation in INS-1 cells. C) Incubation in SF media slightly, but significantly decreased proliferation in MIN6 cells. PGDP had no significant effect on proliferation in MIN6 cells. D) Palmitate (0.2mM) non significantly decreased proliferation in MIN6 cells as compared to serum and 0.5% BSA controls. Pretreatment with PGDP had no effect on proliferation in palmitate treated MIN6 cells. Comparisons in A and C were made relative to SF control, while comparisons in B and D were made relative to 0.2 mM palmitate treatment. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, analyzed with an ANOVA followed by Dunnet's post hoc test. All experiments have sample sizes of $n=8$.

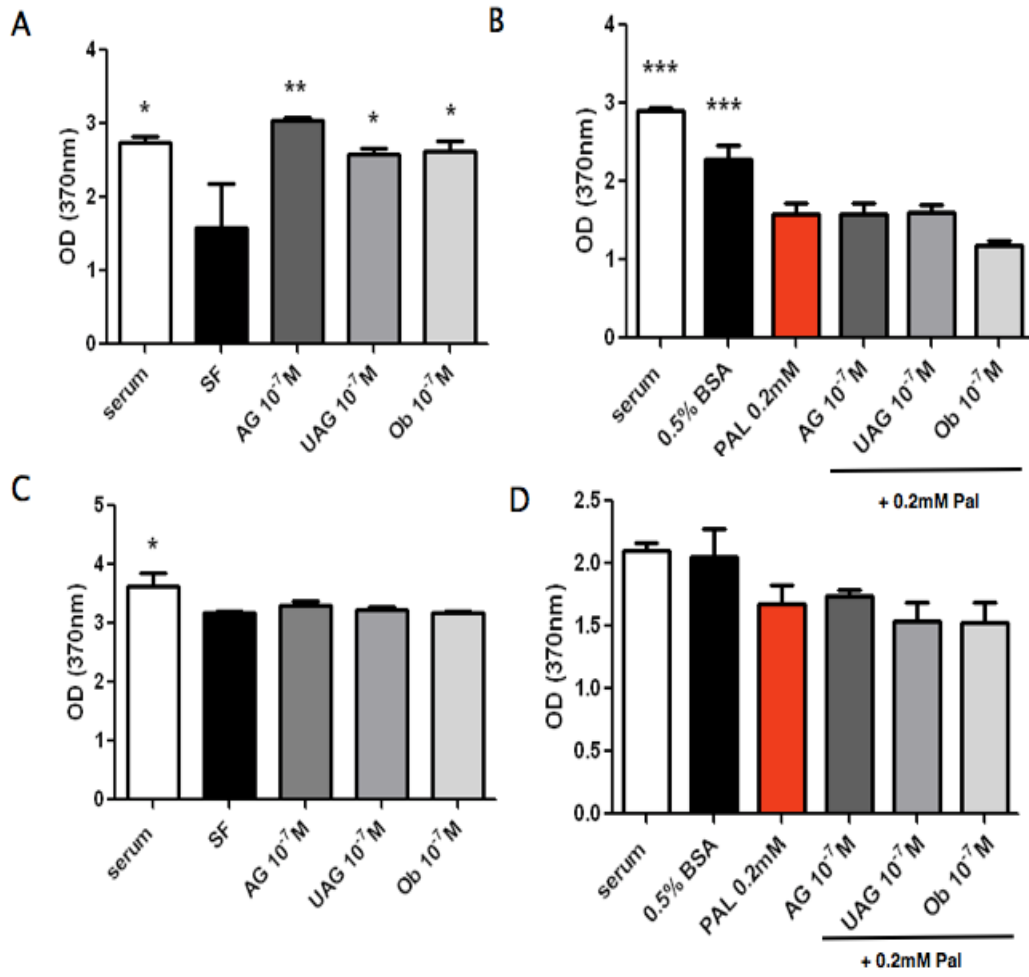


Figure 6

We have found that PGDP increase proliferation in serum starved INS-1 cells. This effect was not found with FFA treated INS-1 cells or with MIN6 cells under any condition.

3.4 PALMITATE INCREASES APOPTOSIS IN INS-1 AND MIN6 CELLS, WHICH IS REDUCED WITH ACYLATED GHRELIN TREATMENT

Despite the lack of strong proliferative effect of PGDP under a lipotoxic environment, the apoptotic rate in response to PGDP and palmitate treatment showed a more pronounced effect. INS-1 and MIN6 cells were incubated overnight in serum, serum free media supplemented with 0.5% FFA free BSA, or 0.2mM palmitate, with or without AG, UAG or obestatin (10^{-7} M). At the end of the 24 hour incubation, cells were incubated with Hoechst nuclear dye in order to visualize nuclei. Condensed and fragmented nuclei were considered apoptotic as apoptotic cells are known to display these characteristics due to condensation of chromatin, DNA fragmentation and formation of apoptotic bodies (Kerr, Wyllie and Currie, 1972).

In INS-1 cells, the percentage of apoptotic cells was increased by palmitate (5.89 ± 0.63 % ($p < 0.001$)) compared to serum and 0.5% BSA controls (0.83 ± 0.26 % and 1.73 ± 0.25 %, respectively) (Figure 7a). AG restored the level of apoptotic cells to serum and 0.5% BSA conditions, with significantly fewer apoptotic cells than when cells were treated with 0.2mM palmitate alone (Figure 7b) (1.79 ± 0.83 % ($p < 0.001$)). UAG was also able to reduce the percentage of apoptotic cells (Figure 7c) (3.27 ± 1.02 % ($p < 0.05$)). Treatment with obestatin did not result in a significant reduction in the percent of apoptotic cells (Figure 7d).

Figure 7. Acylated ghrelin (AG) and unacylated ghrelin (UAG) decrease apoptosis in palmitate treated INS-1 cells. INS-1 cells were plated in chamber slides and grown until 80% confluency. Cells were incubated overnight in serum, serum free media supplemented with 0.5% fatty acid free bovine serum albumin (BSA), or 0.2mM palmitate (prepared as described above) with or without AG, UAG or obestatin (Ob) (10^{-7} M). At the end of the 24 hour incubation, Hoechst nuclear dye 33342 was applied to cells for 15 minutes prior to fixation in 4% PFA. Apoptotic cells were counted in three views of each sample (approximately counts of 100 cells, 40X objective (n=2)). Cells with condensed or fragmented nuclei were considered apoptotic (see arrowheads). Representative view of palmitate treated INS-1 cells (A). Palmitate significantly increased the percent of apoptotic INS-1 cells. (B) AG and (C) UAG co-treatment with 0.2mM palmitate were able to decrease the percent of apoptotic cells, while Ob had no effect (D). Data is expressed as mean % apoptotic cells \pm SEM. * $p < 0.05$, *** $p < 0.001$ compared to 0.2mM palmitate, analyzed with an ANOVA followed by Dunnet's post hoc tests.

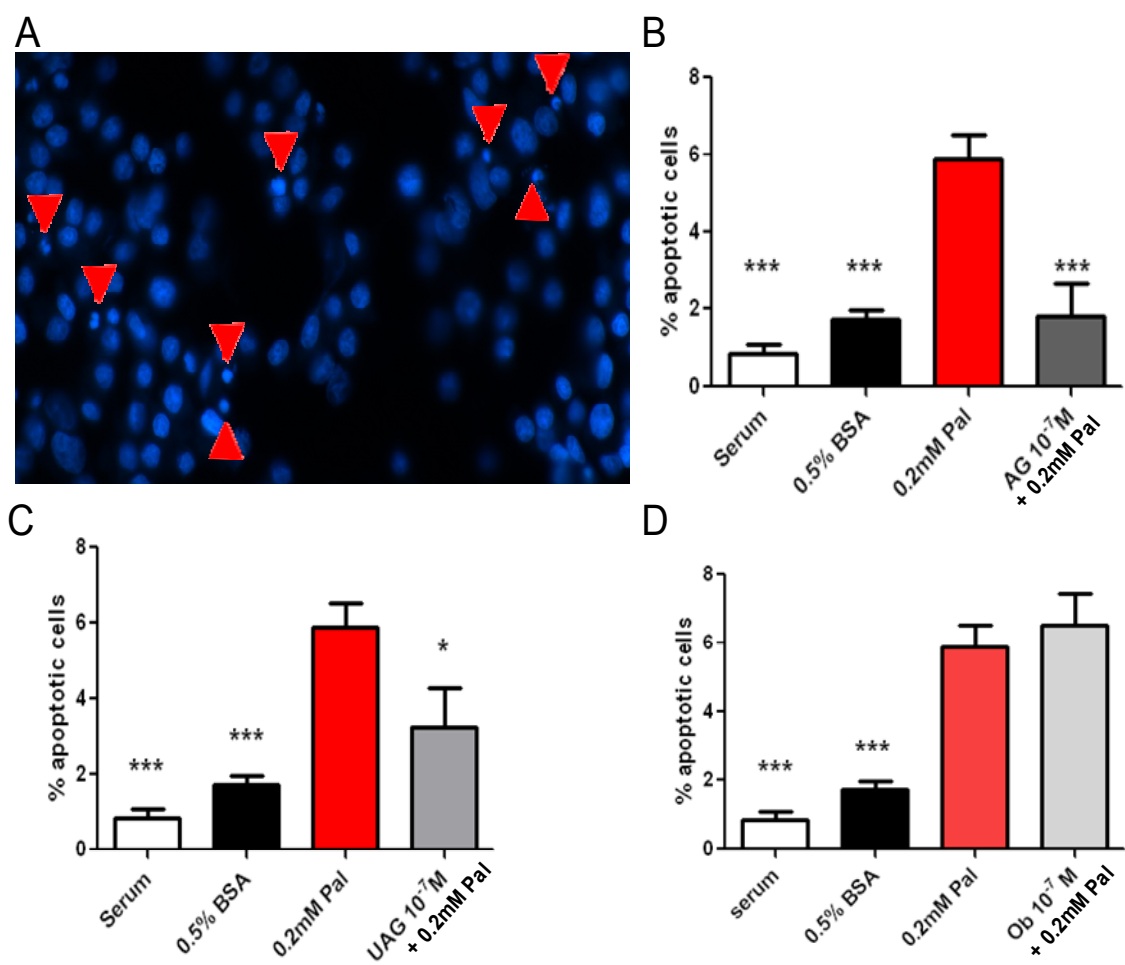


Figure 7

In MIN6 cells, the percentage of apoptotic cells was increased by palmitate ($5.20 \pm 0.50 \%$ ($p < 0.001$)) compared to serum and 0.5% BSA controls ($1.586 \pm 0.20 \%$ and $2.89 \pm 0.25 \%$, respectively) (Figure 8a). AG restored the level of apoptotic cells to serum and 0.5% BSA conditions, with significantly fewer apoptotic cells than wells treated with 0.2mM palmitate alone (Figure 8b) ($3.232 \pm 0.19 \%$ ($p < 0.01$)). Treatment with UAG (Figure 8c) or obestatin (Figure 8d) did not result in a significant reduction in the percent of apoptotic cells.

We have found that palmitate induces apoptosis in INS-1 and MIN6 cells. AG is effective at decreasing the percentage of apoptotic cells in both cell lines, while UAG is only effective at decreasing the percentage of apoptotic cells in INS-1 cells.

3.5 ACTIVATED CASPASE 3 IS ELEVATED IN SERUM DEPRIVED INS-1 CELLS AND IS NORMALIZED WITH ACYLATED GHRELIN TREATMENT

To further explore influence of PGDP on apoptosis in INS-1 and MIN6 cells, activation of the pro-apoptotic protein, caspase 3, was analyzed in 48 hour serum-starved cells incubated in the absence and presence of PDGP. This incubation period was used as we initially found that a 24 hour serum free incubation period was not adequate to increase activated caspase 3 in either cell line. Caspase 3 is a protease, which prior to activation, must be cleaved by caspase 9 (Li et al., 1997). Lysates were incubated with the caspase 3 substrate, acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA); subsequent cleavage of this substrate by caspase 3 results in release of p-nitroaniline, the concentration which was quantified at 405nm.

A 48 hour incubation of INS-1 cells in serum free media led to a significant increase in levels of activated caspase 3 (Figure 9a-c) (OD at 405nm - serum: 0.18 ± 0.01

Figure 8. Acylated ghrelin (AG) decreases apoptosis in palmitate treated MIN6 cells. MIN6 cells were plated in chamber slides and grown until 80% confluency. Cells were incubated overnight in serum, serum free media supplemented with 0.5% fatty acid free bovine serum albumin (BSA), or 0.2mM palmitate (prepared as described above) with or without AG, unacylated ghrelin (UAG) or obestatin (Ob). At the end of the 24 hour incubation, Hoechst nuclear dye 33342 was applied to cells for 15 minutes prior to fixation in 4% PFA. Apoptotic cells were counted in three views of each sample (approximately counts of 100 cells, 40X objective (n=2)). Cells with condensed or fragmented nuclei were considered apoptotic (see arrowheads). Representative view of palmitate treated MIN6 cells (A). Palmitate significantly increased the percent of apoptotic MIN6 cells. AG (B) co-treatment with 0.2mM palmitate was able to decrease the percent of apoptotic cells, while UAG (C) and Ob (D) had no effect. Data is expressed as mean % apoptotic cells \pm SEM. ** $p < 0.01$, *** $p < 0.001$ compared to 0.2mM palmitate, analyzed with an ANOVA followed by Dunnet's post hoc tests.

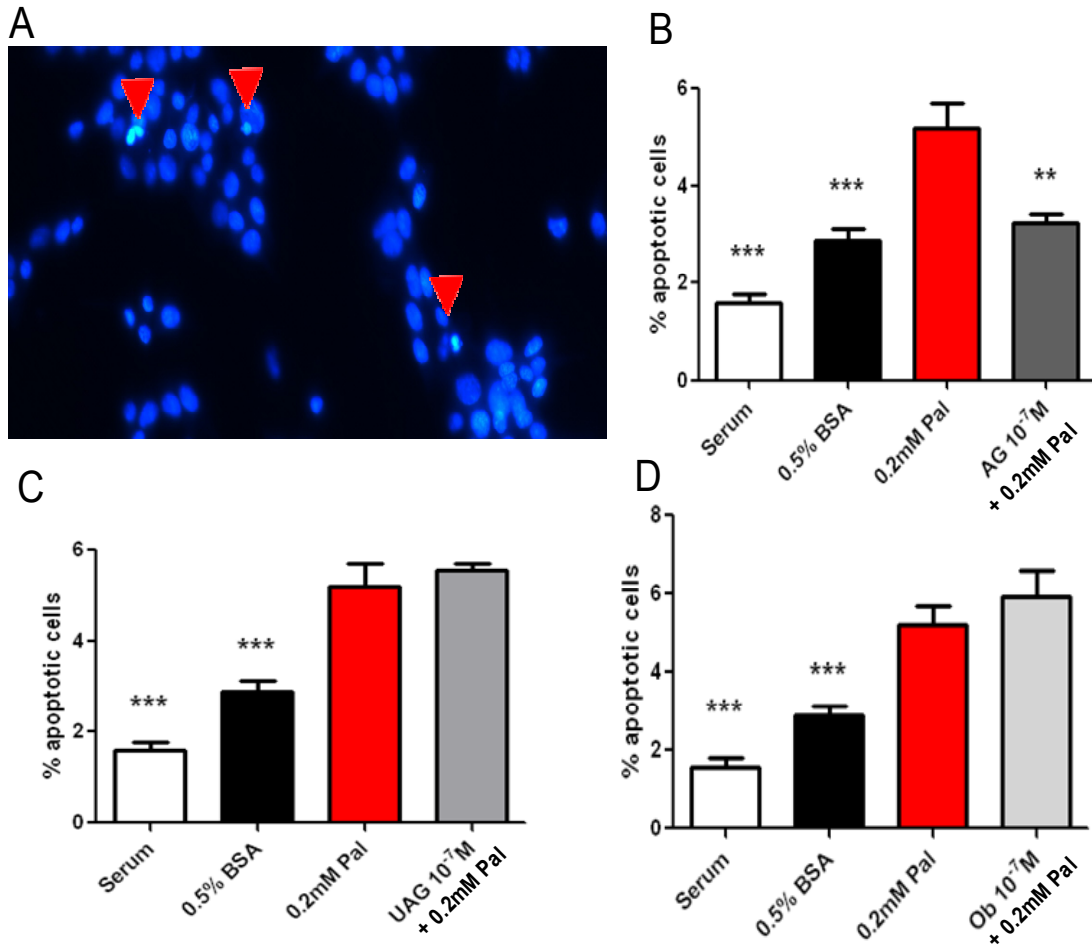
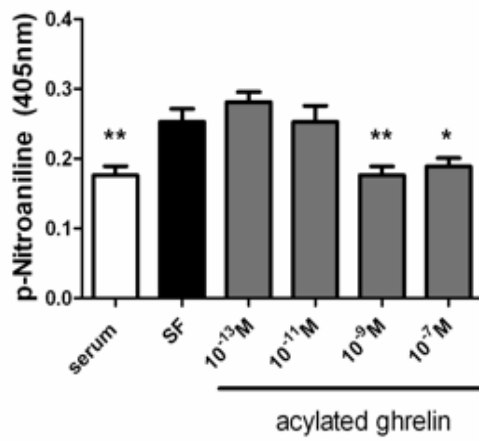


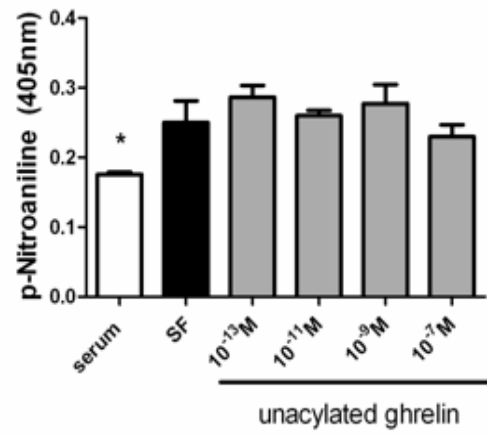
Figure 8

Figure 9. Acylated ghrelin (AG) decreases caspase 3 activation in serum starved INS-1 cells. INS-1 cells were plated in 6 well plates and grown to 80% confluency. INS-1 cells were then incubated for 48 hours in serum free (SF) media with or without AG, unacylated ghrelin (UAG) or Obestatin (Ob) (10^{-13} , 10^{-11} , 10^{-9} , 10^{-7} M). Activated caspase 3 was analyzed in cell lysates with a colorimetric Caspase 3 kit (Sigma, St Louis MO, USA). Data is expressed as mean absorbance at 405nm \pm SEM. Incubation in SF media significantly increased levels of activated caspase 3 as compared to cells incubated in media containing FBS (serum). A) AG (10^{-9} and 10^{-7} M) significantly decreased the amount of caspase 3 present in cell lysate compared to SF cells, while UAG (B) and Ob (C) had no significant effect on caspase 3 activation * $p < 0.05$, ** $p < 0.01$ compared to SF, analyzed with an ANOVA followed by Dunnett's post hoc tests. Samples were performed in triplicate and experiments were completed twice.

A



B



C

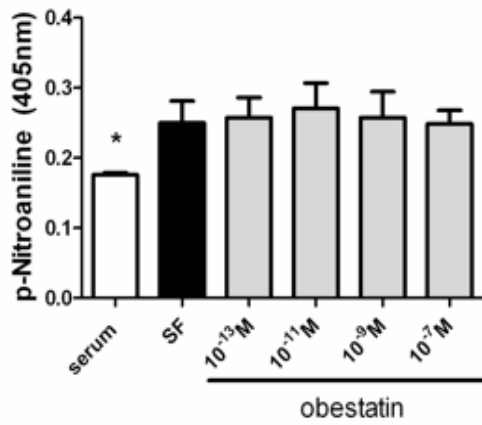


Figure 9

($p < 0.01$) compared to serum free media: 0.25 ± 0.02). Treatment of serum starved cells with AG resulted in a decrease in activated caspase 3, resulting in a restoration of activated caspase 3 levels to that of cells incubated in serum (Figure 9a). This effect was seen at doses of 10^{-9} M AG (OD at 405nm - AG 10^{-9} M: 0.18 ± 0.01 ($p < 0.01$)) and 10^{-7} M AG (AG 10^{-7} M: 0.19 ± 0.01 ($p < 0.05$) compared to serum free media: 0.25 ± 0.02). Activated caspase 3 levels did not change when serum starvation was combined with UAG (Figure 9b) or obestatin (Figure 9c) treatment.

In summary, INS-1 cells have increased levels of activated caspase 3 in response to serum starvation and AG treatment decreases levels of activated caspase 3 to that found in serum treated cells.

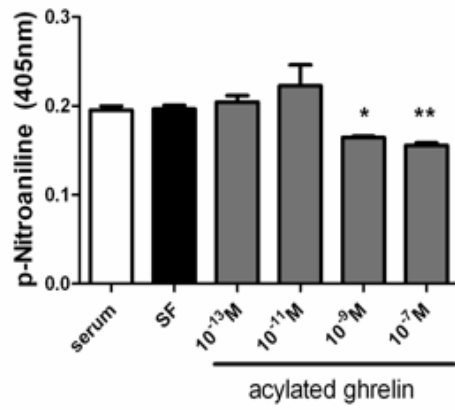
3.6 CASPASE 3 IS NOT ELEVATED IN MIN6 CELLS FOLLOWING 48 HOUR SERUM STARVATION

Serum starvation of MIN6 cells for 48 hours did not increase activated caspase 3 levels as compared with control serum treated cells (Figure 10a-c). Although serum starvation did not induce caspase 3 activity, AG treatment at 10^{-9} M (OD at 405nm - AG 10^{-9} M: 0.16 ± 0.001 ($p < 0.05$)) and 10^{-7} M (AG 10^{-7} M: 0.15 ± 0.002 ($p < 0.01$)) resulted in caspase 3 activation below both serum treated and serum starved cells (Figure 10a) (OD at 405nm - serum: 0.20 ± 0.005 , serum free media: 0.20 ± 0.003). Treatment of cells with UAG (Figure 10b) or obestatin (Figure 10c) did not alter levels of activated caspase 3.

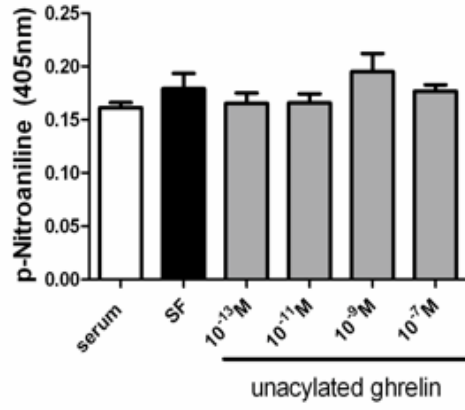
We have found that levels of activated caspase 3 in MIN6 cells are not sensitive to 48 hour serum starvation, but are decreased by AG treatment.

Figure 10. Acylated ghrelin (AG) decreases caspase 3 activation in serum starved MIN6 cells. MIN6 cells were plated in 6 well plates and grown to 80% confluency. MIN6 cells were then incubated for 48 hours in serum free (SF) media with or without AG, unacylated ghrelin (UAG) or Obestatin (Ob) (10^{-13} , 10^{-11} , 10^{-9} , 10^{-7} M). Activated caspase 3 was analyzed in cell lysates with a colorimetric Caspase 3 kit (Sigma, St Louis MO, USA). Data is expressed as mean absorbance at 405nm \pm SEM. Incubation in SF media had no effect on levels of activated caspase 3 as compared to cells incubated in media containing FBS (serum). A) AG (10^{-9} and 10^{-7} M) significantly decreased the amount of caspase 3 present in cell lysate compared to SF cells, while UAG (B) and Ob (C) had no significant effect on activation of caspase 3 * $p < 0.05$, ** $p < 0.01$ compared to SF, analyzed with an ANOVA followed by post hoc tests. Samples were performed in triplicate and experiments were completed twice.

A



B



C

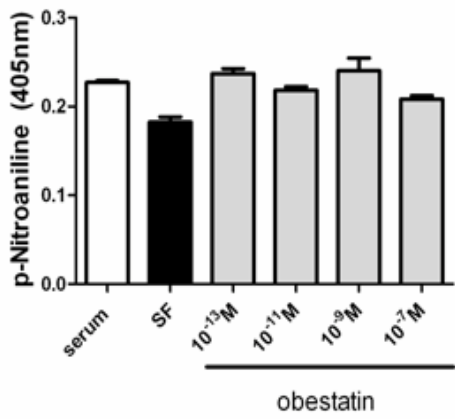


Figure 10

3.7 PALMITATE TREATMENT INCREASES APOPTOSIS IN INS-1 CELLS

The effect of PDGP on FFA induced caspase 3 activation was investigated in INS-1 and MIN6 cells. INS-1 cells treated with 0.2mM palmitate for 24 hours had increased levels of caspase 3 as compared with cells in serum positive and serum free 0.5% BSA conditions (OD at 405nm - serum: 0.17 ± 0.01 , serum free media with 0.5% BSA: 0.18 ± 0.01 (both $p < 0.05$) compared to palmitate treated cells: 0.23 ± 0.01). Co-incubation of cells with AG resulted in a non-significant decrease in active caspase 3 levels (OD at 405nm - AG: 0.21 ± 0.004) (Figure 11). MIN6 cells showed an unstable caspase 3 response to palmitate treatment (data not shown).

We have found levels of caspase 3 in palmitate treated INS-1 cells to be elevated but not sensitive to PGDP treatment.

3.8 ACYLATED GHRELIN AND UNACYLATED GHRELIN INCREASE PHOSPHORYLATION OF AKT IN INS-1 AND MIN6 CELLS

Given the importance of the Akt signaling pathway in the regulation of beta cell mass, phosphorylation of Akt signaling was investigated in INS-1 and MIN6 cells. INS-1 and MIN6 cells were serum starved for 4 hours before a 10 minute incubation with 10^{-7} M AG, UAG or obestatin. Cell lysate was analyzed for phosphorylation of Akt (pAkt) via western blotting. In INS-1 cells, 10^{-7} M AG treatment resulted in a 13% increase in Akt phosphorylation (Figure 12a) (average band density of pAkt normalized to total Akt band density - 10^{-7} M AG: 2.645 ± 0.28 (non significant), compared to serum free control: 2.336 ± 0.15). Furthermore, 10^{-7} M UAG treatment resulted in a 40% increase in Akt phosphorylation (Figure 12b) (average band density of pAkt normalized to total Akt band density - 10^{-7} M UAG: 2.336 ± 0.83 (non significant), compared to serum free control:

Figure 11. Palmitate increases caspase 3 activation INS-1 cells. INS-1 cells were plated in 6 well plates and grown to 80% confluency. INS-1 cells were incubated in 0.2mM palmitate with or without acylated ghrelin (AG), unacylated ghrelin (UAG) or Obestatin (Ob) (10^{-7} M). Activated caspase 3 was analyzed in cell lysates with a colorimetric Caspase 3 kit (Sigma, St Louis MO, USA). Data is expressed as mean absorbance at 405nm \pm SEM. Incubation in 0.2mM palmitate significantly increased levels of activated caspase 3 as compared to cells incubated in media containing FBS (serum) and 0.5% BSA. AG, UAG and Ob had no significant effect on caspase 3 activation * $p < 0.05$ compared to 0.2mM palmitate, analyzed with an ANOVA followed by Dunnet's post hoc tests. Samples were performed in triplicate.

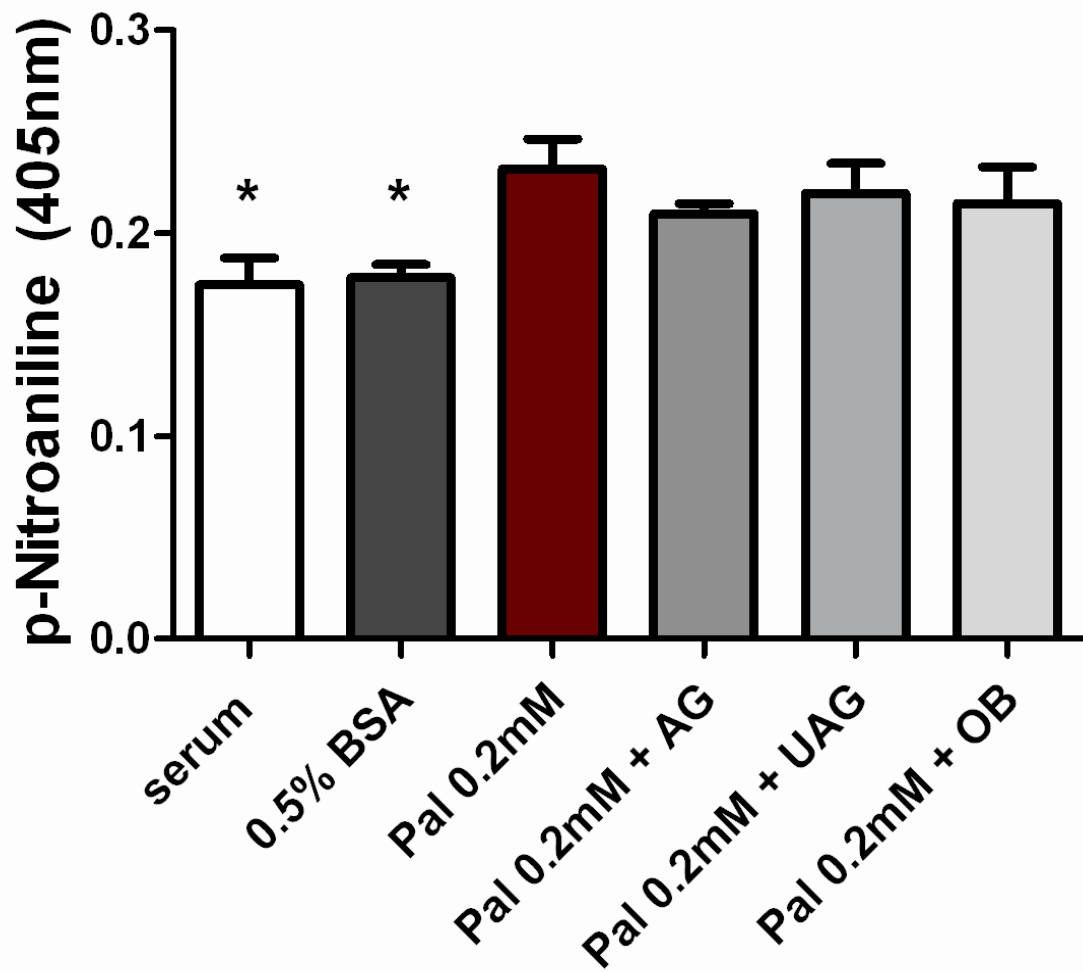


Figure 11

Figure 12. Acylated ghrelin (AG) and unacylated ghrelin (UAG) modestly increase phosphorylation of Akt in INS-1 cells. INS-1 cells were pre-incubated in serum free (SF) media for 4 hours before a 10 minute treatment with proghrelin derived peptides (PGDP) (all treatments 10^{-7} M) ((A) AG, (B) UAG, (C) Obestatin (Ob)). Akt phosphorylation (pAkt) was determined by western blot analysis. Blots were then stripped and re-probed for Akt. Band density was evaluated and expressed as pAkt/Akt. All samples were performed in triplicate and experiments were completed twice.

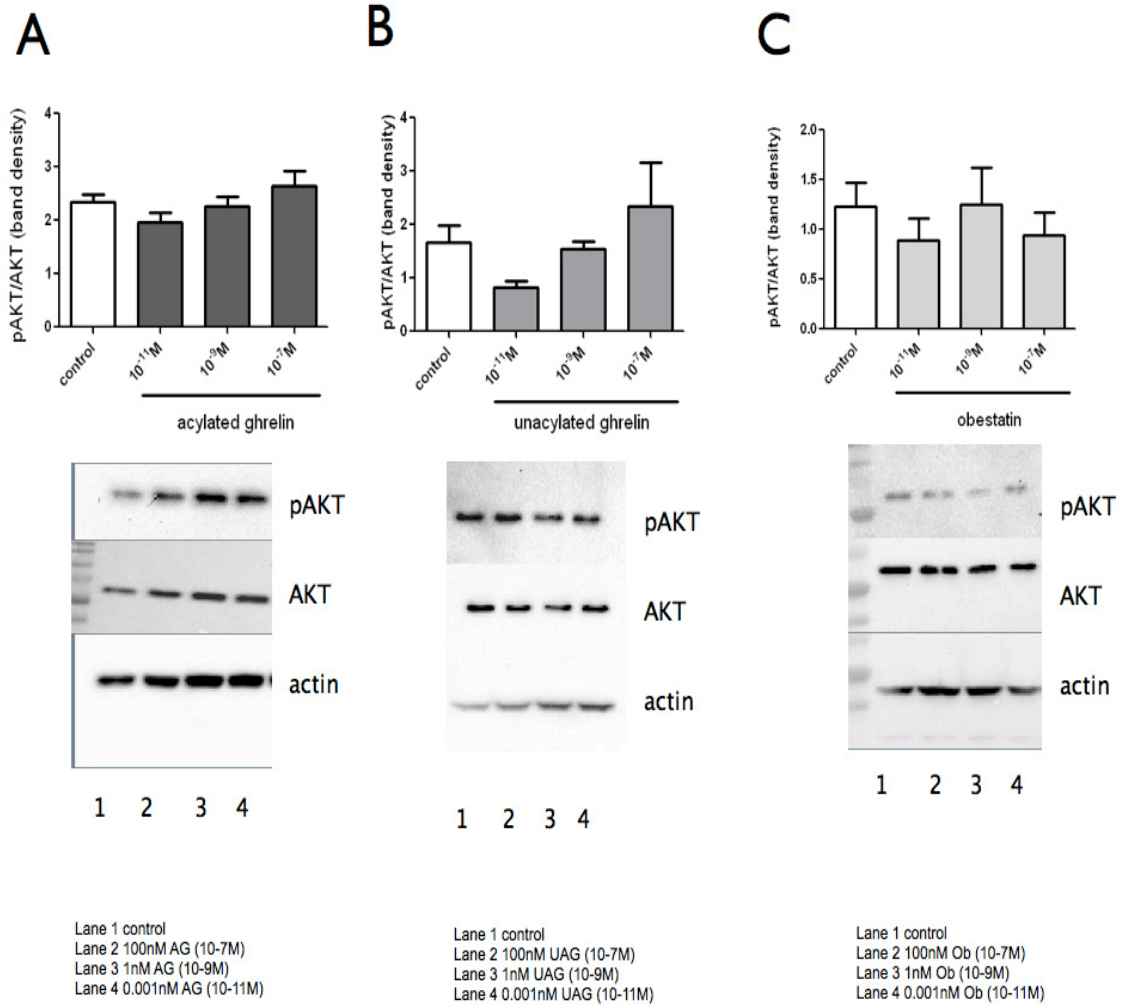


Figure 12

1.67 ± 0.30). In INS-1 cells, obestatin did not increase phosphorylation of Akt (Figure 12c).

In MIN6 cells, 10⁻⁷M AG treatment resulted in a ~30% increase in Akt phosphorylation (Figure 13a) (average band density of pAkt normalized to total Akt band density - 10⁻⁷M AG: 0.0069 ± 0.0003 (non significant), compared to serum free control: 0.0053 ± 0.001). 10⁻⁷M UAG treatment resulted in a 45% increase in Akt phosphorylation (Figure 13b) (average band density of pAkt normalized to total Akt band density - 10⁻⁷M UAG: 0.666 ± 0.12 (non significant), compared to serum free control: 0.459 ± 0.019). In MIN6 cells, obestatin did not increase phosphorylation of Akt (Figure 13c).

We have found that a 10 minute incubation of INS-1 or MIN6 cells with AG or UAG non-significantly increases Akt phosphorylation.

3.9 INSULIN SECRETION IS MODULATED BY PROGHRELIN DERIVED PEPTIDES IN A GLUCOSE DEPENDENT MANNER IN INS-1 CELLS

The ability of PGDP to modulate beta cell function in a cell line lacking GHS R1a was explored by investigating the regulation of insulin secretion by PGDP in INS-1 cells. A background of low, fasting, fed, high and very high glucose concentrations (2, 5, 11, 16 and 25 mM glucose respectively) was used to determine if the PGDP response is glucose dependent. INS-1 cells were incubated for 1 hour in 2, 5, 11, 16 and 25 mM glucose in the presence or absence of AG (Figure 14), UAG (Figure 15) or obestatin (Figure 16). Insulin concentration of the serum was determined with insulin RIA and normalized to total protein content.

Figure 13. Acylated ghrelin (AG) and unacylated ghrelin (UAG) modestly increase phosphorylation of Akt in MIN6 cells. MIN6 cells were pre-incubated in serum free (SF) media for 4 hours before a 10 minute treatment with proghrelin derived peptides (PGDP) (all treatments 10^{-7} M) ((A) AG, (B) UAG, (C) Obestatin (Ob)). Akt phosphorylation (pAkt) was determined by western blot analysis. Blots were then stripped and re-probed for Akt. Band density was evaluated and expressed as pAkt/Akt. All samples were performed in triplicate and experiments were completed twice.

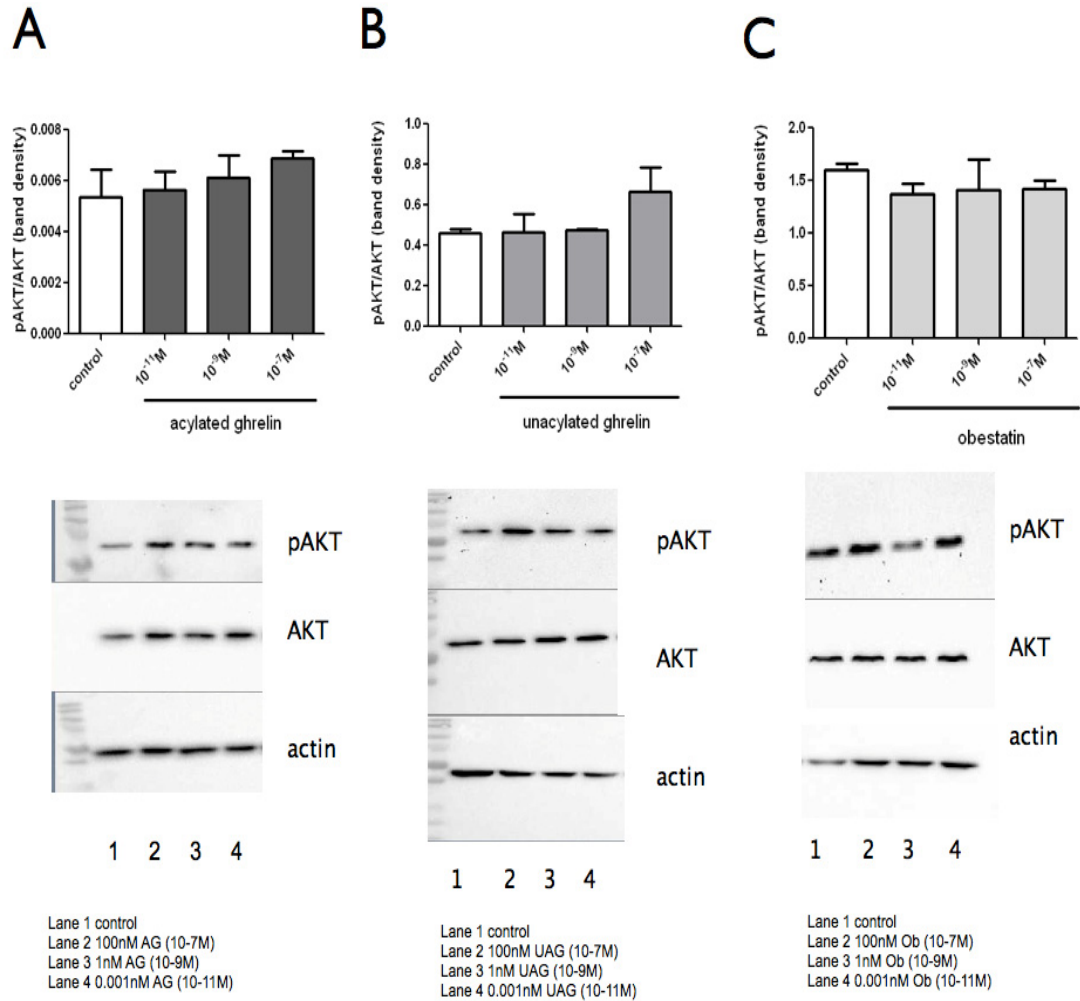


Figure 13

Figure 14. Acylated ghrelin (AG) regulates insulin secretion in INS-1 cells in a glucose and dose dependent manner. INS-1 cells were incubated for 1 hour in 2 (A), 5 (B), 11 (C), 16 (D) and 25mM glucose (E) in the presence or absence of AG (10^{-13} , 10^{-11} , 10^{-9} and 10^{-7} M) (except 25mM glucose which was incubated with AG at 10^{-11} , 10^{-9} and 10^{-7} M). Insulin concentration of the serum was determined with insulin RIA. Insulin concentrations were normalized to total protein content. Data is expressed as mean \pm SEM. At 5mM glucose AG (10^{-13} , 10^{-9} and 10^{-7} M) significantly inhibited insulin secretion (B). Only the lowest dose of AG (10^{-13} M) significantly inhibited insulin secretion at 11mM glucose. * $p < 0.05$ compared to control, analyzed with an ANOVA followed by Dunnet's post hoc tests. Samples were performed in triplicate and experiments with completed twice.

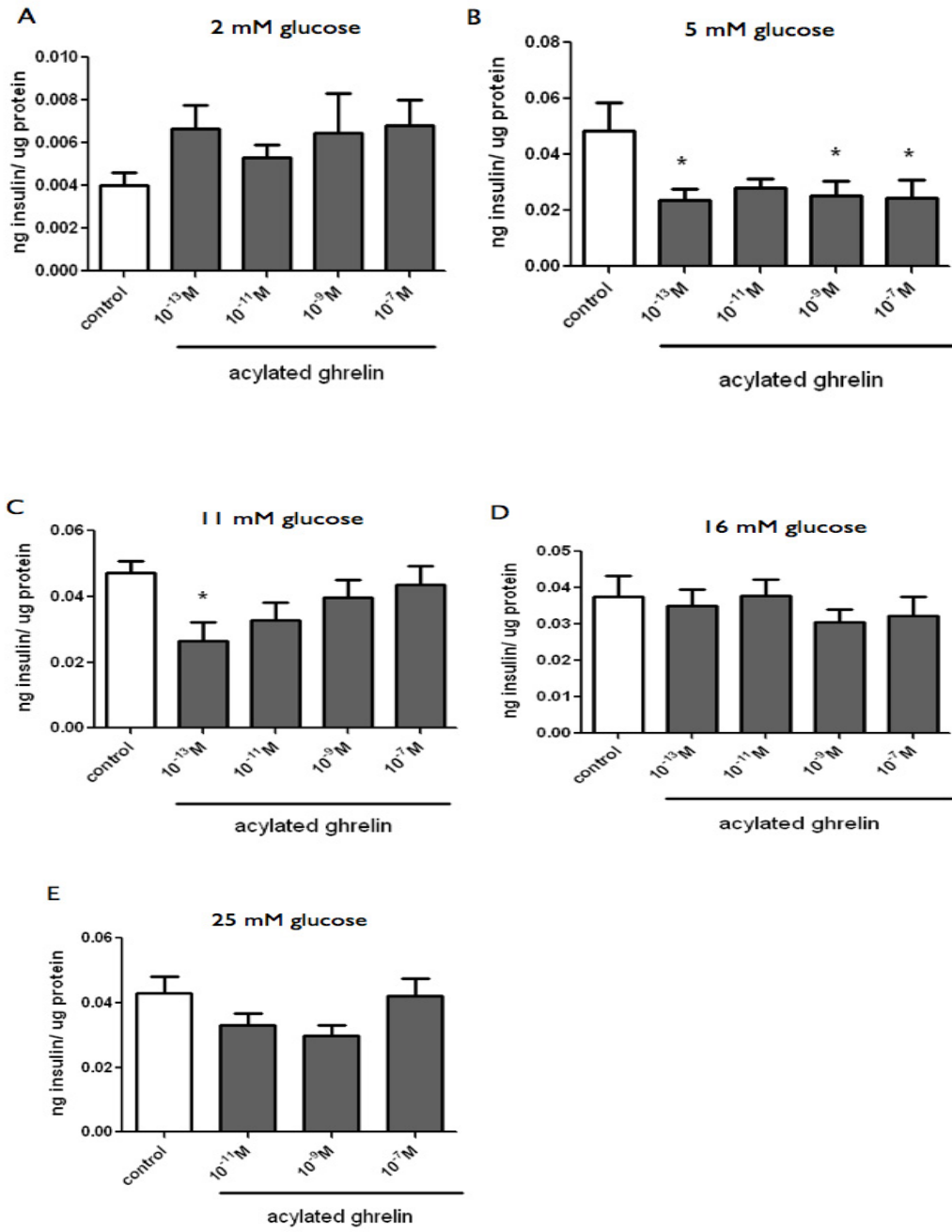


Figure 14

Figure 15. Unacylated ghrelin (UAG) regulates insulin secretion in INS-1 cells in a glucose and dose dependent manner. INS-1 cells were incubated for 1 hour in 2 (A), 5 (B), 11 (C), 16 (D) and 25mM glucose (E) in the presence or absence of UAG (10^{-13} , 10^{-11} , 10^{-9} and 10^{-7} M) (except 25mM glucose which was incubated with UAG at 10^{-11} , 10^{-9} and 10^{-7} M). Insulin concentration of the serum was determined with insulin RIA. Insulin concentrations were normalized to total protein content. Data is expressed as mean \pm SEM. At 2mM glucose UAG (10^{-9} M) significantly increased insulin secretion (A). At 5mM glucose UAG (10^{-11} and 10^{-9} M) significantly inhibited insulin secretion (B). At 16mM glucose UAG (10^{-11} M) significantly increased insulin secretion (D). * $p < 0.05$, ** $p < 0.01$ compared to control, analyzed with an ANOVA followed by Dunnet's post hoc tests. Samples were performed in triplicate and experiments were completed twice.

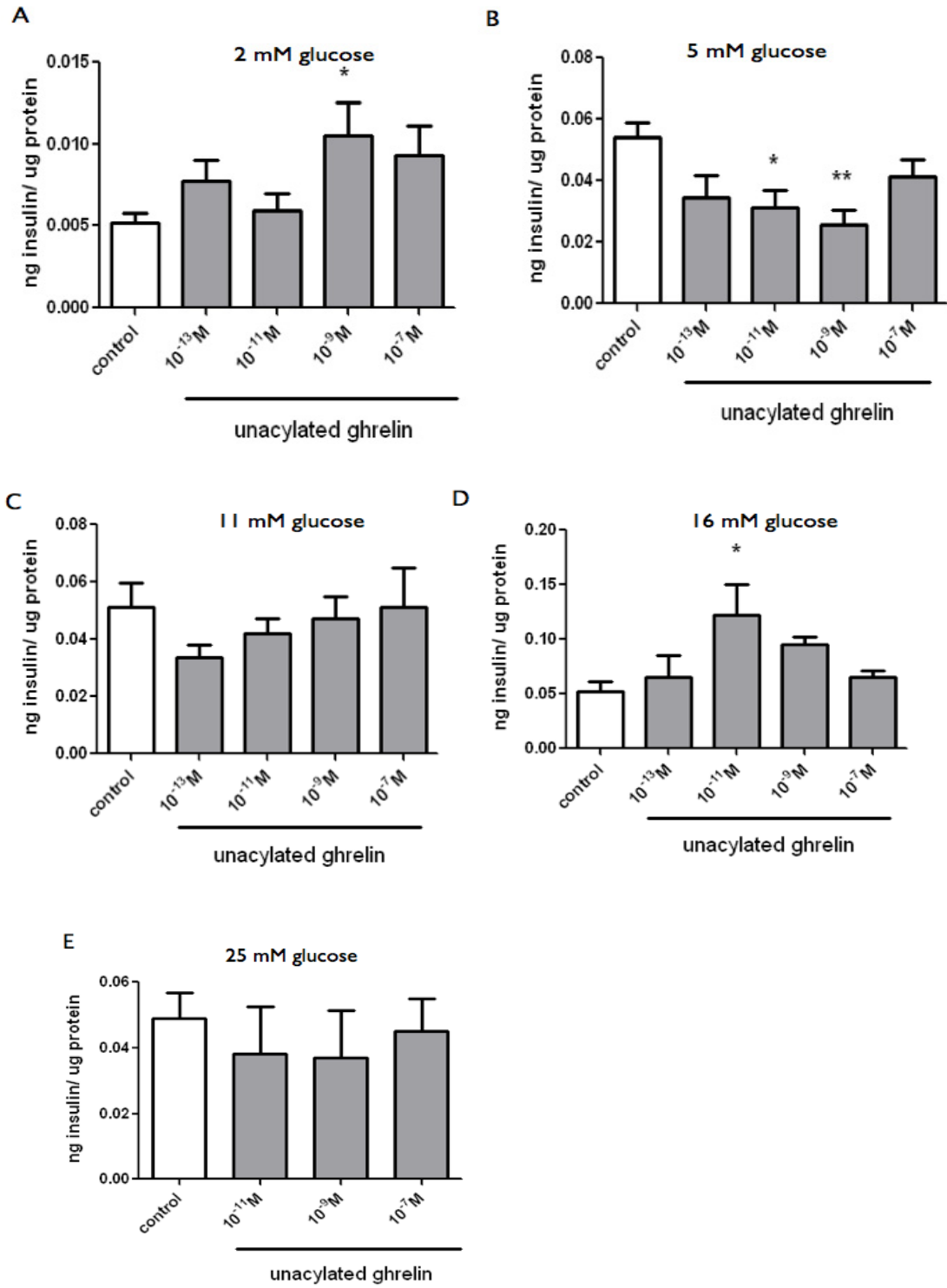


Figure 15

Figure 16. Obestatin (Ob) regulates insulin secretion in INS-1 cells in a glucose and dose dependent manner. INS-1 cells were incubated for 1 hour in 2 (A), 5 (B), 11 (C), 16 (D) and 25mM glucose (E) in the presence or absence of Ob (10^{-13} , 10^{-11} , 10^{-9} and 10^{-7} M) (except 25mM glucose which was incubated with Ob at 10^{-11} , 10^{-9} and 10^{-7} M). Insulin concentration of the serum was determined with insulin RIA. Insulin concentrations were normalized to total protein content. Data is expressed as mean \pm SEM. At 2mM glucose, the lowest dose of Ob (10^{-13} M) significantly increased insulin secretion (A). At 11mM glucose, the three lowest doses of Ob (10^{-13} , 10^{-11} and 10^{-9} M) significantly inhibited insulin secretion (C). At 16mM glucose Ob (10^{-11} M) significantly increased insulin secretion (D). * $p < 0.05$, ** $p < 0.01$ compared to control, analyzed with an ANOVA followed by Dunnett's post hoc tests. Samples were performed in triplicate and experiments were completed twice.

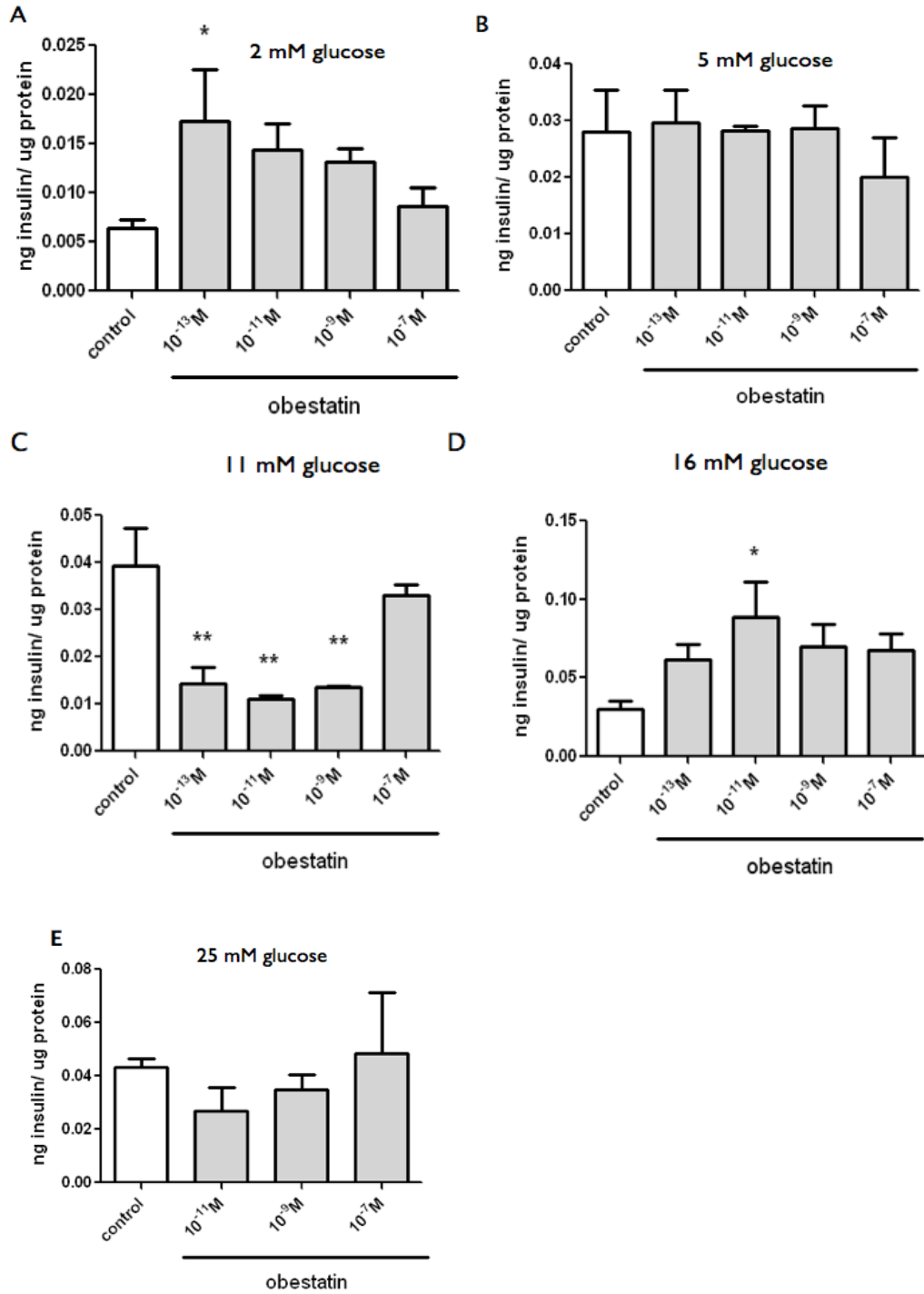


Figure 16

3.9.1 Acylated Ghrelin Inhibits Insulin Secretion

AG treatment of INS-1 cells leads to inhibition of insulin secretion in a glucose and dose dependent manner. While no significant effect of AG was found at 2 (Figure 14a), 16 (Figure 14d) or 25 mM glucose (Figure 14e), AG resulted in significant inhibition of insulin secretion at doses of 10^{-13} M, 10^{-9} M, and 10^{-7} M at 5 mM glucose (AG: 10^{-13} M: 0.023 ± 0.004 ng insulin/ μ g protein ($p < 0.05$), 10^{-9} M: 0.025 ± 0.005 ng insulin/ μ g protein ($p < 0.05$) and 10^{-7} M: 0.024 ± 0.006 ng insulin/ μ g protein ($p < 0.05$), compared to control (0.048 ± 0.01 ng insulin/ μ g protein) (Figure 14b). At a concentration of 11mM glucose, only the lowest tested dose of ghrelin was found to inhibit insulin secretion (AG: 10^{-13} M: 0.026 ± 0.006 ng insulin/ μ g protein ($p < 0.05$), compared to control (0.047 ± 0.003 ng insulin/ μ g protein) (Figure 14c).

3.9.2 Unacylated Ghrelin Inhibits and Stimulates Insulin Secretion

UAG also produced inhibition of insulin secretion at 5mM glucose, with doses of 10^{-11} M and 10^{-9} M AG significantly decreasing insulin secretion (AG: 10^{-11} M: 0.03 ± 0.006 ng insulin/ μ g protein ($p < 0.05$), 10^{-9} M: 0.026 ± 0.005 ng insulin/ μ g protein ($p < 0.01$), compared to control (0.053 ± 0.005 ng insulin/ μ g protein) (Figure 15b). Unlike AG, UAG produced significant stimulation of insulin secretion at 2mM glucose (AG: 10^{-9} M: 0.01 ± 0.002 ng insulin/ μ g protein ($p < 0.05$), compared to control (0.005 ± 0.0006 ng insulin/ μ g protein)) (Figure 15a), and at 16mM glucose (AG: 10^{-11} M: 0.12 ± 0.028 ng insulin/ μ g protein ($p < 0.05$), compared to control (0.052 ± 0.008 ng insulin/ μ g protein)) (Figure 15d).

3.9.3 Obestatin Inhibits and Stimulates Insulin Secretion

Obestatin produced inhibition of insulin secretion at 11 mM glucose, with doses of 10^{-13} M, 10^{-11} M and 10^{-9} M obestatin significantly decreasing insulin secretion (Ob: 10^{-13} M: 0.014 ± 0.003 ng insulin/ μ g protein ($p < 0.01$), 10^{-11} M: 0.011 ± 0.0008 ng insulin/ μ g protein ($p < 0.01$), and 10^{-9} M: 0.014 ± 0.0003 ng insulin/ μ g protein ($p < 0.01$) compared to control (0.040 ± 0.008 ng insulin/ μ g protein) (Figure 16c). Similar to UAG, obestatin produced significant stimulation of insulin secretion at 2mM glucose (Ob: 10^{-13} M: 0.017 ± 0.005 ng insulin/ μ g protein ($p < 0.05$), compared to control (0.0063 ± 0.001 ng insulin/ μ g protein)) (Figure 16a), and at 16mM glucose (Ob: 10^{-11} M: 0.089 ± 0.023 ng insulin/ μ g protein ($p < 0.05$), compared to control (0.030 ± 0.006 ng insulin/ μ g protein)) (Figure 16d).

CHAPTER 4 DISCUSSION

4.1 FURTHER EVIDENCE FOR UNKNOWN PROGHRELIN DERIVED PEPTIDE RECEPTORS

It has become evident that a number of unknown receptors exist for PGDP. Actions of AG have been reported in tissues and cells known to not express GHS R1a, indicating that an additional receptor for AG is present in at least some tissues (Granata et al., 2007, Johansson et al., 2008, De Vriese et al., 2005, Delhanty et al., 2006). The identity of this receptor is currently beyond speculation. UAG has no known receptor (Camina, 2006, Soares and Leite-Moreira, 2008), although UAG has bioactivity in a number of tissues and cell lines (Granata et al., 2007, Chen et al., 2005, Toshinai et al., 2006, Gauna et al., 2006). The possibilities remain that there exists a single unknown receptor that recognizes both AG and UAG, or a number of receptors that are mutually exclusive between AG and UAG. Obestatin was originally reported as the ligand for GPR39 (Zhang et al., 2005); however this has recently been questioned (Depoortere et al., 2008, Unniappan, Speck and Kieffer, 2008, Tang et al., 2008, Lauwers et al., 2006, Kobelt et al., 2008a). Thus, obestatin may also have an unknown receptor, although it is clear from our results that obestatin does possess biological activities in the beta cell. The identity of these unknown receptor(s) is vital to the understanding of ghrelin and its associated peptide hormones.

Here we report the effects of PGDP on proliferation and survival in two beta cell lines, one which expresses GHS R1a and one which does not. We have confirmed the presence of GHS R1a in MIN6 cells (Colombo et al., 2003, Doi et al., 2006) and shown that INS-1 cells lack GHS R1a mRNA and protein. Using these beta cell models we were

able to investigate the action of PGDP on cell lines with or lacking this traditional ghrelin receptor. Furthermore, both MIN6 and INS-1 cells are widely used models of pancreatic beta cells due to their ability to secrete insulin in a glucose dependent fashion (Asfari et al., 1992, Ishihara et al., 1993). Although cell lines allow greater ease of manipulation for culture based experiments, they are not representative the environment of beta cells in islets, where cells are surrounded by other endocrine cell types. As such, caution should be used in interpreting results obtained by cell lines. However, given the complexity of the ghrelin system in the endocrine pancreas, we believe that experiments in cell lines are necessary to establish the basics of ghrelin action and unknown receptors before moving on to the more complex setting of whole islet preparations.

4.1.1 Acylated Ghrelin Decreases Apoptosis in the Presence and Absence of GHS R1a

In both cell lines, AG promoted cell survival as measured by both a decrease in the percentage of cells with an apoptotic phenotype (Figure 7 & 8) and a decrease in the amount of active caspase 3 (Figure 9 & 10). The ability of AG to affect cell survival in a model lacking GHS R1a was not unexpected as previous reports of AG bioactivity have been reported in cells without GHS R1a (Granata et al., 2007, Johansson et al., 2008, DeVriese et al., 2005, Delhanty et al., 2006). Indeed, our results provide further support to the idea that there are additional receptors for AG.

4.1.2 INS-1 Cells Lack GHS R1a and Respond Differently to Acylated Ghrelin than Their Clonal Derivatives

Our finding of INS-1 cells lacking GHS R1a is novel. There are reports of low expression of GHS R1a in the clonal derivatives of INS-1 cells, INS-1E (Colombo et al.,

2003) and INS-1 (832/13) (Wierup et al., 2004). However, given their differences in stability and insulin secretion (Oreana et al., 2008, Hohmeier et al., 2000), discrepancies between these cell lines are not unexpected. Furthermore, the effect of AG on insulin secretion in both INS-1E and INS-1 (832/13) cells differs from the effect reported by our group, suggesting that these two cell lines respond differently to ghrelin, a finding which would be supported by expression of different AG receptors. AG (1nM) treatment in INS-1E cells has been reported to result in inhibition of insulin secretion only under conditions of 16.7 and 25mM glucose (Colombo et al., 2003), while inhibition of insulin secretion is evident in INS-1 (832/13) cells only with 100nM AG treatment at 15mM glucose. In contrast, our results show that insulin secretion in INS-1 cells is unresponsive to AG at 16 and 25mM glucose (Figure 14). These variances ghrelin response in INS-1 derived cell lines is most likely due to differences in their complement of PGDP receptors.

4.1.3 Acylated Ghrelin and Unacylated Ghrelin Increase Cell Number in the Presence and Absence of GHS R1a

In both INS-1 and MIN6 cells, AG and UAG were effective at maintaining cell number under conditions of serum starvation (Figure 4 & 5). The receptor through which this action is mediated is currently unclear. Although MIN6 cells express GHS R1a, this does not exclude the possibility that these cells may also express an additional ghrelin receptor, such as that present in INS-1 cells. Furthermore, obestatin was capable of enhancing beta cell mass in MIN6 cells only, suggesting that, although INS-1 cells are able to respond to obestatin (Figure 16), the regulation of cell number in this cell line is not sensitive to obestatin.

4.2 PROGHRELIN DERIVED PEPTIDES REGULATE BETA CELL MASS IN INS-1 AND MIN6 CELLS

Beta cell mass is determined by the combined effects of beta cell size, proliferation, neogenesis and beta cell death (Rhodes, 2005). As cell number is an ambiguous measure of beta cell mass we further investigated the role of PGDP on beta cell size, proliferation and survival. Preliminary results suggested that neither AG, UAG or obestatin effected the average beta cell size (Figure 4 & 5), so our focus shifted to the regulation of proliferation and survival.

4.2.1 Effects of PGDP on Proliferation

Rates of proliferation were first tested in INS-1 and MIN6 cells under conditions of serum deprivation. While proliferation of INS-1 cells was sensitive to serum starvation, MIN6 cells were more resistant to this stressor (Figure 6). To determine if an effect of PGDP was masked by decreased sensitivity of MIN6 to serum deprivation, rates of proliferation were tested under the more harsh condition of prolonged incubation with the free fatty acid palmitate.

In conditions of serum starvation, PGDP induced proliferation in INS-1 cells. Serum starved INS-1 cell showed significantly decreased rates of proliferation and treatment with AG, UAG or obestatin returned rates of proliferation to that of serum treated control cells (Figure 6a). MIN6 cells were much less sensitive to serum starvation, as evidenced by only a slight, but significant, decline in proliferation rates (Figure 6c). Perhaps as a result of this decreased sensitivity to serum deprivation, PGDP were unable to produce a significant change in proliferation in serum starved MIN6 cells.

Therefore, it appears that the increase in beta cell mass in INS-1 cells in response to AG and UAG is at least partially due to rates of increased proliferation. The increased rate of proliferation in response to obestatin in INS-1 cells was somewhat unexpected, given that obestatin treatment did not increase cell number (figure 4f). It remains possible that the increased rate of proliferation seen with obestatin treatment in INS-1 cells is balanced by a corresponding loss of cells due to increased apoptosis or weakened attachments to culture plates. Furthermore, our results suggest that the mechanism by which PGDP increase cell number in MIN6 cells is not due to increases in cellular proliferation.

Treatment of INS-1 cells with palmitate resulted in significant decreases in proliferation, although under these lipotoxic conditions, PGDP were ineffective at restoring cellular proliferation (Figure 6b). Proliferation rates in MIN6 cells were surprisingly unresponsive to palmitate, with the decline of cellular proliferation due to palmitate treatment not reaching statistical significance (Figure 6d). PGDP treatment had no effect on proliferation rates of MIN6 cells exposed to palmitate.

The similarity of proliferation rates to both serum deprivation and palmitate treatment in MIN6 cells suggests that proliferation in this cell line is very resistant to metabolic stressors. However, palmitate has been reported to decrease proliferation of MIN6 cells by 32% (Wang et al., 2010b), and this decline in proliferation has been shown to be sensitive to AG treatment (Wang et al., 2010b). The reasons for the differences between these results and ours are not clear. Variations in experimental protocols for palmitate exposure or BrdU incorporation time periods may be responsible for the variance between our work and others. However, we tested PGDP both at the beginning

of palmitate treatment (data not shown) and as a pretreatment to subsequent co-treatment with palmitate (Figure 6), finding neither protocol effective at increasing proliferation. In addition, by setting the BrdU incorporation period to the full length of the palmitate treatment, we report a clearer description of proliferation of the entire experiment. Although this time period for BrdU incorporation limits our ability to detect bursts in proliferation due to PGDP, the overall impact on proliferation over the entire time period is of greater value to determining the mechanisms responsible for alterations in cell number over a prolonged treatment time. Other reports of AG increasing proliferation of MIN6 during a 24 hour palmitate treatment used a BrdU incorporation period of one hour (Wang et al., 2010b), the reported findings may indicate a brief or transient phenomenon, rather than our longer term investigation of proliferation rate.

The differences in PGDP sensitivity in INS-1 cells between serum deprivation and lipotoxicity suggests that the signaling pathways activated by PGDP to rescue proliferation in serum starved cells are either different from that needed to stimulate proliferation in palmitate treated cells, or is simply too weak of a signal to compensate for lipotoxicity.

4.2.2 Effects of PGDP on Cell Survival

The effect of PGDP on cell survival was investigated in both INS-1 and MIN6 cells. We first examined cells for the induction of apoptosis via microscopy. Given the relatively low sensitivity of this method for detecting early apoptosis signaling, we chose to test cells with the strong inducer of apoptosis, palmitate (Maestre et al., 2003). Although these experiments do not relate directly back to our preliminary results on cell

number, they were performed to look at cell survival under a harsh and physiologically relevant stressor to beta cells. Exposure to lipotoxic conditions in both cell lines (24 hour treatment with palmitate) produced a significant increase in the percentage of cells with an apoptotic phenotype (condensed or fragmented nuclei) (Figure 7 & 8). A reduction in the percentage of apoptotic cells with both AG and UAG was found in INS-1 cells, while only AG was able to decrease the percentage of apoptotic cells in MIN6 cells. Obestatin had no effect on the percentage of apoptotic cells in either cell line.

As mentioned previously, the disadvantage of the analysis of cells for phenotypic markers of apoptosis is the potential to overlook the earlier stages of apoptosis that occur at the level of cell signaling prior to the morphological nuclear changes. Indeed, while in preliminary experiments, serum deprivation (48 hours) was unable to significantly effect the morphological characteristics of either cell line (data not shown), earlier steps in the apoptotic cascade were evident in INS-1 cells (Figure 9). Measurement of the amount of activated caspase 3, an effector molecule in the classical apoptosis signaling cascade (Nicholson et al., 1995, Wen et al., 1997, Kothakota et al., 1997, Porter and Janicke, 1999, Fernandes-Alnemri, Litwack and Alnemri, 1994), indicated that treatment of serum deprived INS-1 cells with AG was able to return the levels of active caspase 3 to that of serum treated control cells (Figure 9a). In line with the decreased sensitivity of MIN6 cells to changes in proliferation, levels of active caspase 3 did not significantly increase with 48 hours of serum deprivation. However, AG was still able to produce a decrease in levels of activated caspase 3 in this cell line, with levels of active caspase 3 decreasing below both serum deprived and serum treated control cells. In both INS-1 and MIN6 cells neither UAG or obestatin altered the amount of active caspase 3.

4.2.3 Summary of PGDP Effects on Beta Cell Mass in INS-1 Cells

These findings suggest that there is a different mechanism for the AG and UAG stimulated increase in INS-1 cell number in serum deprived cells. AG treatment triggers both an increase in proliferation and a decrease in activation of the pro-apoptotic signaling pathway (cleavage of caspase 3), while UAG treatment increases cellular proliferation but has no effect on caspase 3 activation. How these differences between AG and UAG occur is currently unclear. As we have shown, INS-1 cells lack the traditional ghrelin receptor, GHS R1a. Our results show that AG is capable of eliciting effects independent of UAG in this cell line, indicating that AG is able to induce cell signaling that is not due to degradation of AG into UAG. It is unclear whether these effects occur via one receptor with binding for both AG and UAG, or through two distinct novel ghrelin receptors. Future experiments on competitive binding with AG and UAG radioligands will serve to shed light on these results.

4.2.4 Summary of PGDP Effects on Beta Cell Mass in MIN6 Cells

The enhancement of cell number in AG treated serum starved MIN6 cells appears to be due the reduction in caspase 3 activity in MIN6 cells, and therefore in decreased rates of apoptosis. While it is most plausible that this effect occurs via GHS R1a, MIN6 cells may also express additional receptors for AG of unknown identity. While there are GHS R1a antagonists available, the specificity of the binding of GHS has been questioned (Johansson et al., 2008, Cassoni et al., 2001). Therefore, molecular techniques involving deletion of GHS R1a in MIN6 cells would provide clarification on this matter. However, the increase in cell number in this cell line may also occur via apoptotic independent mechanisms, as the effective dose range for AG in the cell number

experiments (10^{-13} M, 10^{-11} M, 10^{-9} and 10^{-7} M) is greater than that for the caspase 3 experiments (10^{-9} and 10^{-7} M). Other possible mechanisms for enhancement of cell number need to be explored including a more complete investigation of proliferation and survival signaling pathways to determine the how lower doses of AG increase cell number. The mechanism whereby UAG and obestatin increase in beta cell number in MIN6 cells is unclear. Neither increases in proliferation or decreases in caspase 3 levels were found with either peptide. There remains the possibility that the rate of apoptosis is decreased in MIN6 cells after UAG or obestatin treatment in a manner which is not reflected by decreased caspase 3 activation. Indeed, apoptosis can be triggered via pathways that are independent of caspase 3 (Porter and Janicke, 1999, Janicke et al., 1998). Further investigation on the activation of these pathways by UAG and obestatin in MIN6 cells would aid in the understanding of PGDP regulation of beta cell mass in this cell line.

4.2.5 Lipotoxicity and Apoptosis

Caspase 3 activation was further tested under conditions of palmitate induced lipotoxicity. While palmitate incubation increased levels of activated caspase 3 in INS-1 cells, co-treatment with PDGP did not alleviate this effect (Figure 11). This result mirrors that seen with palmitate and decreased proliferation in this cell line; either PGDP do not activate the proper signaling cascades to rescue cells from palmitate treatment, or their signal is too weak to compensate for lipotoxicity induced caspase 3 activation. Caspase 3 activation was also analyzed in palmitate treated MIN6 cells; however the response of this cell line was highly unstable and therefore not reported here. Although we found palmitate to induce an apoptotic phenotype in this cell line, it is not clear why

we were unable to find increased activation of caspase 3 levels in MIN6 cells. However, other groups have reported increased caspase 3 cleavage after prolonged palmitate treatment in MIN6 cells (Wang et al., 2010b, Wang et al., 2010a).

4.2.6 INS-1 and MIN6 Cells Differ in Their Sensitivity to Metabolic Stressors

The difference between INS-1 and MIN6 cells in sensitivity to serum deprivation and fatty acid treatment was not unexpected. INS-1 cells have been reported to be more sensitive to palmitate treatment than MIN6 cells (Lai et al., 2008). The reasons for this effect are unclear, but most likely due to differences in specific proteins present in these cell lines. The difference in palmitate sensitivity was suggested to be due to elevated levels of the enzyme stearoyl-coenzyme A desaturase-1 (Lai et al., 2008). Differences in GHS R1a could also play a role in the differences between MIN6 and INS-1 cells. Not only do MIN6 cells express GHS R1a, but this cell line has also been reported to express ghrelin, indicating the ability of this cell line to produce and secrete ghrelin (Nakashima et al., 2008). The likely autocrine role of ghrelin in MIN6 cells may be involved in the increased resistance to metabolic stressors in this cell line.

4.2.7 Activation of Akt Signaling in INS-1 and MIN6 Cells

PGDP have been reported to stimulate proliferation and enhance survival in a variety of beta cell models via PI3K/Akt signaling (Granata et al., 2007, Granata et al., 2008, Zhang et al., 2007). Our preliminary results on PGDP Akt activation agree with these reports. Both AG and UAG produced a dose dependent, but non-significant increase in Akt phosphorylation in INS-1 and MIN6 cells (Figure 12 &13). Expansion of the experimental protocol to include time points other than 10 minutes may allow us to see stronger evidence of Akt signaling in cell lines. Akt activation was not stimulated by

10 minute treatment with obestatin in either INS-1 or MIN6 cells. While it may be that obestatin does not possess Akt signaling in these cell lines, the length of treatment is another plausible reason for the lack of effect of obestatin. Obestatin does enhance Akt phosphorylation HIT-T15 and INS-1E beta cell lines (Granata et al., 2008), however it remains to be seen if this is the case in INS-1 or MIN6 cells. Further work is required to investigate these matters. PGDP have also been shown to increase cellular proliferation and survival by activation of MAPK signaling (Granata et al., 2007, Granata et al., 2008, Zhang et al., 2007). It is possible that PGDP signal via MAPK in INS-1 and MIN6 cells to increase beta cell mass. Future work should explore the roles of Akt and MAPK signaling in the mediation of PGDP effects.

4.3 PROGHRELIN DERIVED PEPTIDES MODULATE INSULIN SECRETION IN INS-1 CELLS

PGDP have been reported as modulators of insulin secretion both *in vivo* and *in vitro*. However, the results to date are inconsistent, with variations across experimental models and protocols. We chose to explore the effect of PGDP on insulin secretion in INS-1 cells to determine the effect of ghrelin in a system lacking the traditional ghrelin receptor. INS-1 cells were tested across a full physiological dose range of PGDP and against different background glucose concentrations.

4.3.1 Ghrelin Regulation of Insulin Secretion

AG affected insulin secretion in a glucose dependent manner (Figure 14), with a one hour AG treatment significantly inhibiting insulin secretion at 5mM glucose at nearly all doses of AG. Intuitively, these results appear to fit with the fluctuations of AG with respect to energy state. AG concentrations in circulation are the highest during periods of

fasting (Tschop, Smiley and Heiman, 2000, Guo et al., 2008), a time when the release of insulin must be decreased to maintain normoglycemia. Our results suggest that one of the physiological roles of ghrelin in the endocrine pancreas is to contribute to dampening of insulin secretion from beta cells during periods of fasting. We have also shown that a reverse dose response curve for AG inhibition of insulin secretion occurs at 11mM glucose. It is currently unclear why only a 0.1 pM dose of AG is able to inhibit insulin secretion under 11mM glucose. Given that INS-1 cells possess a novel ghrelin receptor, little is known on the kinetics of this unknown receptor. The traditional ghrelin receptor, GHS R1a, is reported to have high rates of receptor internalization (Camina et al., 2004), and such a phenomenon would explain the dose response curve seen with AG treatment at 11mM glucose in INS-1 cells.

Insulin secretion in INS-1 cells is also affected by UAG (Figure 15). In a similar fashion to AG, UAG inhibited insulin secretion at 5mM, across nearly all doses and although not statistically significant, 0.1pM UAG inhibited insulin secretion at 11mM glucose. The parallels between these results suggest that both AG and UAG are acting through the same receptor or signaling pathways to achieve these results. Interestingly, UAG has additional effects on insulin secretion from those shared with AG, with stimulation of insulin secretion at 1nM UAG at 2mM glucose and 0.01nM UAG at 16mM glucose. The discrepancies between AG and UAG actions on insulin secretion in INS-1 cells suggest that AG is not simply degrading into UAG. Currently unclear is the number and identity of receptors responsible for these effects on insulin secretion in INS-1 cells. The similarity in the inhibitory profiles of AG and UAG suggests that both AG and UAG may bind to the same receptor to mediate these effects or simply two separate

receptors with similar signaling mechanisms. The ability of UAG to stimulate insulin secretion at specific doses in a glucose dependent manner is an aspect of UAG signaling that is unique from AG. UAG may activate another receptor to induce this stimulation or may cause different receptor coupling than AG. As with the regulation of beta cell mass, the roles and signaling pathways of ghrelin in insulin secretion may be clarified by competitive binding experiments.

Our reports of inhibition of insulin secretion by AG support a solely inhibitory role of AG in insulin regulation. UAG was found to produce inhibition of insulin secretion at similar doses and glucose concentrations to AG. Many reports of AG causing inhibition of insulin secretion are found in the literature (Wierup et al., 2004, Dezaki et al., 2006, Esler et al., 2007, Salehi et al., 2004, Qader et al., 2008, Reimer, Pacini and Ahren, 2003, Qader et al., 2005, Dezaki et al., 2004, Dezaki, Kakei and Yada, 2007, Colombo et al., 2003, Wang et al., 2010c), and our results support these findings. Differences in the dose or glucose concentration at which AG is maximally effective are most likely due to model or species differences. We are the first to report an inhibitory action of UAG on insulin secretion. As acylation of ghrelin has been reported to occur in the Golgi prior to processing of proghrelin (Zhu et al., 2006, Yang et al., 2008), it is unlikely that this effect of UAG is due to transformation into AG. We have also found UAG to stimulate insulin secretion at 2 and 16mM glucose. These results are supported by previous reports of UAG stimulating insulin secretion under similar conditions (Granata et al., 2007). It is currently unclear why some groups have found stimulation of insulin secretion by AG. Given the results of the current work, it remains possible that

other reports of AG stimulation of insulin secretion could be due to degradation of AG into UAG. Model or species differences may also account for these differences.

4.3.2 Obestatin Regulation of Insulin Secretion

Obestatin also modulated insulin secretion in a glucose dependent manner in INS-1 cells (Figure 16). At 2mM glucose, obestatin treatment stimulated insulin secretion, in a reverse dose response pattern, with only 0.1pM obestatin reaching statistical significance. Obestatin also stimulated insulin secretion at a dose of 0.01nM at 16mM glucose. Inhibition of insulin secretion was found at 0.1pM-1nM at 11mM glucose. These findings are in agreement with previous reports of obestatin inhibiting insulin secretion at 8.3mM glucose in perfused rat pancreas (Qader et al., 2008). Granata et al. (2008), reported that 100nM obestatin stimulates insulin secretion from human islets at 0, 2, 7.5, 15 and 25 mM glucose, findings that are partially supported by our results at 2 and 16mM glucose. Differential regulation of insulin secretion by obestatin across a range of glucose concentrations was been reported before (Egido et al., 2009), however our findings differ from these in the reported response pattern. The discrepancies between our results and others is most likely due to differences in the models used (perfused pancreas or islets versus cell lines) and species differences. The greater sensitivity of INS-1 cells to the lower range of obestatin doses suggests that, like the unknown ghrelin receptor, the receptor for obestatin may also be subjected to receptor desensitization.

Variable responses of PGDP on insulin secretion have been reported by others (Salehi et al., 2004, Qader et al., 2008). The reasons for differing responses of PGDP across variable glucose concentrations indicates there is interplay between glucose and PGDP signaling.

4.4 CONCLUSION

Our results present a complicated picture of PGDP regulation of beta cell mass and function. The presence of multiple unknown receptors makes it difficult to speculate the interactions between PGDP and their receptors, although it is clear that the predicted variations make for a system that is highly flexible but tightly controlled.

Ghrelin is regulated by feeding state, being elevated during both short (Tschop, Smiley and Heiman, 2000, Guo et al., 2008) and long term (Otto et al., 2001, Broglio et al., 2004, Kinzig, Hargrave and Tao, 2009, Yang et al., 2007) states of energy shortages, therefore one of the functions of ghrelin in the pancreas could be the regulation of beta cell mass (maintenance) as well as the inhibition of insulin secretion at a time when blood glucose is low. In this capacity, ghrelin acts to stabilize blood glucose (preventing insulin induced hypoglycemia) as well as protecting the population of beta cells. As such, the actions of ghrelin may be most beneficial to type 1 diabetics or as pharmaceutical enhancement given to type 2 diabetics during fasting to decrease insulin resistance triggered hyperinsulinemia and prevent damage to the current pool of beta cells, possibly slowing the progression of the disease.

It is also possible that pancreatic PGDP play their most important role during times of metabolic change. As ghrelin levels fluctuate with energy state (Tschop et al., 2001, Erdmann et al., 2005, Otto et al., 2001, Broglio et al., 2004, Kinzig, Hargrave and Tao, 2009, Yang et al., 2007, Lee et al., 2002), ghrelin concentration provides input to the pancreas on the level of dietary shortages or excesses, information which may be used to determine if beta cell mass should be modified.

Future research on PGDP and the pancreas should focus further on characterizing the receptors for PGDP and the resultant signaling pathways that are activated upon PGDP binding. Although complex, the ability of PGDP to modulate both beta cell mass and function may prove useful in the treatment of diabetes.

REFERENCES

- Ackermann AM, Gannon M (2007) Molecular regulation of pancreatic beta-cell mass development, maintenance, and expansion. *J Mol Endocrinol* 38:193-206.
- Adeghate E, Ponery AS (2002) Ghrelin stimulates insulin secretion from the pancreas of normal and diabetic rats. *J Neuroendocrinol* 14:555-560.
- Alvarez CJ, Lodeiro M, Theodoropoulou M, Camina JP, Casanueva FF, Pazos Y (2009) Obestatin stimulates akt signalling in gastric cancer cells through beta-arrestin-mediated epidermal growth factor receptor transactivation. *Endocr Relat Cancer* 16:599-611.
- Andrews ZB, Liu ZW, Wallingford N, Erion DM, Borok E, Friedman JM, Tschop MH, Shanabrough M, Cline G, Shulman GI, Coppola A, Gao XB, Horvath TL, Diano S (2008) UCP2 mediates ghrelin's action on NPY/AgRP neurons by lowering free radicals. *Nature* 454:846-851.
- Asakawa A, Inui A, Kaga T, Katsuura G, Fujimiya M, Fujino MA, Kasuga M (2003) Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. *Gut* 52:947-952.
- Asakawa A, Inui A, Fujimiya M, Sakamaki R, Shinfuku N, Ueta Y, Meguid MM, Kasuga M (2005) Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. *Gut* 54:18-24.
- Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Nijima A, Fujino MA, Kasuga M (2001) Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 120:337-345.
- Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB (1992) Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130:167-178.
- Baggio LL, Drucker DJ (2007) Biology of incretins: GLP-1 and GIP. *Gastroenterology* 132:2131-2157.
- Bagnasco M, Tulipano G, Melis MR, Argiolas A, Cocchi D, Muller EE (2003) Endogenous ghrelin is an orexigenic peptide acting in the arcuate nucleus in response to fasting. *Regul Pept* 111:161-167.
- Balcazar N, Sathyamurthy A, Elghazi L, Gould A, Weiss A, Shiojima I, Walsh K, Bernal-Mizrachi E (2009) mTORC1 activation regulates beta-cell mass and proliferation by modulation of cyclin D2 synthesis and stability. *J Biol Chem* 284:7832-7842.

Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonisconi S, Fubini A, Malan D, Baj G, Granata R, Broglio F, Papotti M, Surico N, Bussolino F, Isgaard J, Deghenghi R, Sinigaglia F, Prat M, Muccioli G, Ghigo E, Graziani A (2002) Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. *J Cell Biol* 159:1029-1037.

Banks WA, Burney BO, Robinson SM (2008) Effects of triglycerides, obesity, and starvation on ghrelin transport across the blood-brain barrier. *Peptides* 29:2061-2065.

Banks WA, Tschop M, Robinson SM, Heiman ML (2002) Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther* 302:822-827.

Bednarek MA, Feighner SD, Pong SS, McKee KK, Hreniuk DL, Silva MV, Warren VA, Howard AD, Van Der Ploeg LH, Heck JV (2000) Structure-function studies on the new growth hormone-releasing peptide, ghrelin: Minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. *J Med Chem* 43:4370-4376.

Bernal-Mizrachi E, Wen W, Stahlhut S, Welling CM, Permutt MA (2001) Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. *J Clin Invest* 108:1631-1638.

Bernal-Mizrachi E, Fatrai S, Johnson JD, Ohsugi M, Otani K, Han Z, Polonsky KS, Permutt MA (2004) Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced akt activity in pancreatic islet beta cells. *J Clin Invest* 114:928-936.

Bernard-Kargar C, Ktorza A (2001) Endocrine pancreas plasticity under physiological and pathological conditions. *Diabetes* 50 Suppl 1:S30-5.

Bouwens L, Rooman I (2005) Regulation of pancreatic beta-cell mass. *Physiol Rev* 85:1255-1270.

Broglio F, Gottero C, Prodam F, Gauna C, Muccioli G, Papotti M, Abribat T, Van Der Lely AJ, Ghigo E (2004b) Non-acylated ghrelin counteracts the metabolic but not the neuroendocrine response to acylated ghrelin in humans. *J Clin Endocrinol Metab* 89:3062-3065.

Broglio F, Arvat E, Benso A, Gottero C, Muccioli G, Papotti M, van der Lely AJ, Deghenghi R, Ghigo E (2001) Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. *J Clin Endocrinol Metab* 86:5083-5086.

Broglio F, Gianotti L, Destefanis S, Fassino S, Abbate Daga G, Mondelli V, Lanfranco F, Gottero C, Gauna C, Hofland L, Van der Lely AJ, Ghigo E (2004a) The endocrine

response to acute ghrelin administration is blunted in patients with anorexia nervosa, a ghrelin hypersecretory state. *Clin Endocrinol (Oxf)* 60:592-599.

Budihardjo I, Oliver H, Lutter M, Luo X, Wang X (1999) Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* 15:269-290.

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC (2003) Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102-110.

Cai Y, Wang Q, Ling Z, Pipeleers D, McDermott P, Pende M, Heimberg H, Van de Casteele M (2008) Akt activation protects pancreatic beta cells from AMPK-mediated death through stimulation of mTOR. *Biochem Pharmacol* 75:1981-1993.

Camina JP (2006) Cell biology of the ghrelin receptor. *J Neuroendocrinol* 18:65-76.

Camina JP, Campos JF, Caminos JE, Dieguez C, Casanueva FF (2007) Obestatin-mediated proliferation of human retinal pigment epithelial cells: Regulatory mechanisms. *J Cell Physiol* 211:1-9.

Camina JP, Carreira MC, El Messari S, Llorens-Cortes C, Smith RG, Casanueva FF (2004) Desensitization and endocytosis mechanisms of ghrelin-activated growth hormone secretagogue receptor 1a. *Endocrinology* 145:930-940.

Cassoni P, Ghe C, Marrocco T, Tarabra E, Allia E, Catapano F, Deghenghi R, Ghigo E, Papotti M, Muccioli G (2004) Expression of ghrelin and biological activity of specific receptors for ghrelin and des-acyl ghrelin in human prostate neoplasms and related cell lines. *Eur J Endocrinol* 150:173-184.

Cassoni P, Papotti M, Ghe C, Catapano F, Sapino A, Graziani A, Deghenghi R, Reissmann T, Ghigo E, Muccioli G (2001) Identification, characterization, and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines. *J Clin Endocrinol Metab* 86:1738-1745.

Chan CB, Saleh MC, Koshkin V, Wheeler MB (2004) Uncoupling protein 2 and islet function. *Diabetes* 53 Suppl 1:S136-42.

Chanoine JP, Wong AC (2004) Ghrelin gene expression is markedly higher in fetal pancreas compared with fetal stomach: Effect of maternal fasting. *Endocrinology* 145:3813-3820.

Chanoine JP, Wong AC, Barrios V (2006) Obestatin, acylated and total ghrelin concentrations in the perinatal rat pancreas. *Horm Res* 66:81-88.

- Chen CY, Inui A, Asakawa A, Fujino K, Kato I, Chen CC, Ueno N, Fujimiya M (2005) Des-acyl ghrelin acts by CRF type 2 receptors to disrupt fasted stomach motility in conscious rats. *Gastroenterology* 129:8-25.
- Choi D, Woo M (2010) Executioners of apoptosis in pancreatic β -cells: Not just for cell death. *Am J Physiol Endocrinol Metab* 298:E735-41.
- Chung H, Kim E, Lee DH, Seo S, Ju S, Lee D, Kim H, Park S (2007) Ghrelin inhibits apoptosis in hypothalamic neuronal cells during oxygen-glucose deprivation. *Endocrinology* 148:148-159.
- Colombo M, Gregersen S, Xiao J, Hermansen K (2003) Effects of ghrelin and other neuropeptides (CART, MCH, orexin A and B, and GLP-1) on the release of insulin from isolated rat islets. *Pancreas* 27:161-166.
- Cowley MA et al (2003) The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 37:649-661.
- Cunha SR, Mayo KE (2002) Ghrelin and growth hormone (GH) secretagogues potentiate GH-releasing hormone (GHRH)-induced cyclic adenosine 3',5'-monophosphate production in cells expressing transfected GHRH and GH secretagogue receptors. *Endocrinology* 143:4570-4582.
- Currie PJ, Mirza A, Fuld R, Park D, Vasselli JR (2005) Ghrelin is an orexigenic and metabolic signaling peptide in the arcuate and paraventricular nuclei. *Am J Physiol Regul Integr Comp Physiol* 289:R353-R358.
- Date Y, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, Kojima M, Kangawa K, Arima T, Matsuo H, Yada T, Matsukura S (2002) Ghrelin is present in pancreatic α -cells of humans and rats and stimulates insulin secretion. *Diabetes* 51:124-129.
- De Smet B, Depoortere I, Moechars D, Swennen Q, Moreaux B, Cryns K, Tack J, Buyse J, Coulie B, Peeters TL (2006) Energy homeostasis and gastric emptying in ghrelin knockout mice. *J Pharmacol Exp Ther* 316:431-439.
- De Vriese C, Gregoire F, De Neef P, Robberecht P, Delporte C (2005) Ghrelin is produced by the human erythroleukemic HEL cell line and involved in an autocrine pathway leading to cell proliferation. *Endocrinology* 146:1514-1522.
- Delhanty PJ, van der Eerden BC, van der Velde M, Gauna C, Pols HA, Jahr H, Chiba H, van der Lely AJ, van Leeuwen JP (2006) Ghrelin and unacylated ghrelin stimulate human osteoblast growth via mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase (PI3K) pathways in the absence of GHS-R1a. *J Endocrinol* 188:37-47.

- Depoortere I, Thijs T, Moechars D, De Smet B, Ver Donck L, Peeters TL (2008) Effect of peripheral obestatin on food intake and gastric emptying in ghrelin-knockout mice. *Br J Pharmacol* 153:1550-1557.
- Dezaki K, Sone H, Yada T (2008) Ghrelin is a physiological regulator of insulin release in pancreatic islets and glucose homeostasis. *Pharmacol Ther* 118:239-249.
- Dezaki K, Kakei M, Yada T (2007) Ghrelin uses Galphai2 and activates voltage-dependent K⁺ channels to attenuate glucose-induced Ca²⁺ signaling and insulin release in islet beta-cells: Novel signal transduction of ghrelin. *Diabetes* 56:2319-2327.
- Dezaki K, Hosoda H, Kakei M, Hashiguchi S, Watanabe M, Kangawa K, Yada T (2004) Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca²⁺ signaling in beta-cells: Implication in the glycemic control in rodents. *Diabetes* 53:3142-3151.
- Dezaki K, Sone H, Koizumi M, Nakata M, Kakei M, Nagai H, Hosoda H, Kangawa K, Yada T (2006) Blockade of pancreatic islet-derived ghrelin enhances insulin secretion to prevent high-fat diet-induced glucose intolerance. *Diabetes* 55:3486-3493.
- Dickson LM, Rhodes CJ (2004) Pancreatic beta-cell growth and survival in the onset of type 2 diabetes: A role for protein kinase B in the akt? *Am J Physiol Endocrinol Metab* 287:E192-8.
- Doi A, Shono T, Nishi M, Furuta H, Sasaki H, Nanjo K (2006) IA-2beta, but not IA-2, is induced by ghrelin and inhibits glucose-stimulated insulin secretion. *Proc Natl Acad Sci U S A* 103:885-890.
- Dor Y, Brown J, Martinez OI, Melton DA (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 429:41-46.
- Doyle ME, Egan JM (2007) Mechanisms of action of glucagon-like peptide 1 in the pancreas. *Pharmacol Ther* 113:546-593.
- Egido EM, Hernandez R, Marco J, Silvestre RA (2009) Effect of obestatin on insulin, glucagon and somatostatin secretion in the perfused rat pancreas. *Regul Pept* 152:61-66.
- Elghazi L, Bernal-Mizrachi E (2009) Akt and PTEN: Beta-cell mass and pancreas plasticity. *Trends Endocrinol Metab* 20:243-251.
- Elghazi L, Balcazar N, Bernal-Mizrachi E (2006) Emerging role of protein kinase B/Akt signaling in pancreatic beta-cell mass and function. *Int J Biochem Cell Biol* 38:157-163.
- Elghazi L, Rachdi L, Weiss AJ, Cras-Meneur C, Bernal-Mizrachi E (2007) Regulation of beta-cell mass and function by the Akt/protein kinase B signalling pathway. *Diabetes Obes Metab* 9 Suppl 2:147-157.

- Erdmann J, Lippl F, Wagenpfeil S, Schusdziarra V (2005) Differential association of basal and postprandial plasma ghrelin with leptin, insulin, and type 2 diabetes. *Diabetes* 54:1371-1378.
- Esler WP, Rudolph J, Claus TH, Tang W, Barucci N, Brown SE, Bullock W, Daly M, Decarr L, Li Y, Milardo L, Molstad D, Zhu J, Gardell SJ, Livingston JN, Sweet LJ (2007) Small-molecule ghrelin receptor antagonists improve glucose tolerance, suppress appetite, and promote weight loss. *Endocrinology* 148:5175-5185.
- Fatrai S, Elghazi L, Balcazar N, Cras-Meneur C, Krits I, Kiyokawa H, Bernal-Mizrachi E (2006) Akt induces beta-cell proliferation by regulating cyclin D1, cyclin D2, and p21 levels and cyclin-dependent kinase-4 activity. *Diabetes* 55:318-325.
- Fernandes-Alnemri T, Litwack G, Alnemri ES (1994) CPP32, a novel human apoptotic protein with homology to caenorhabditis elegans cell death protein ced-3 and mammalian interleukin-1 beta-converting enzyme. *J Biol Chem* 269:30761-30764.
- Franke TF (2008) PI3K/Akt: Getting it right matters. *Oncogene* 27:6473-6488.
- Franke TF, Hornik CP, Segev L, Shostak GA, Sugimoto C (2003) PI3K/Akt and apoptosis: Size matters. *Oncogene* 22:8983-8998.
- Gauna C, van de Zande B, van Kerkwijk A, Themmen AP, van der Lely AJ, Delhanty PJ (2007) Unacylated ghrelin is not a functional antagonist but a full agonist of the type 1a growth hormone secretagogue receptor (GHS-R). *Mol Cell Endocrinol* 274:30-34.
- Gauna C, Delhanty PJ, van Aken MO, Janssen JA, Themmen AP, Hofland LJ, Culler M, Broglio F, Ghigo E, van der Lely AJ (2006) Unacylated ghrelin is active on the INS-1E rat insulinoma cell line independently of the growth hormone secretagogue receptor type 1a and the corticotropin releasing factor 2 receptor. *Mol Cell Endocrinol* 251:103-111.
- Glauser DA, Schlegel W (2007) The emerging role of FOXO transcription factors in pancreatic beta cells. *J Endocrinol* 193:195-207.
- Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, Korbonits M (2002) The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J Clin Endocrinol Metab* 87:2988.
- Gonzalez CR, Vazquez MJ, Lopez M, Dieguez C (2008) Influence of chronic undernutrition and leptin on GOAT mRNA levels in rat stomach mucosa. *J Mol Endocrinol* 41:415-421.
- Granata R, Settanni F, Gallo D, Trovato L, Biancone L, Cantaluppi V, Nano R, Annunziata M, Campiglia P, Arnoletti E, Ghe C, Volante M, Papotti M, Muccioli G,

Ghigo E (2008) Obestatin promotes survival of pancreatic beta-cells and human islets and induces expression of genes involved in the regulation of beta-cell mass and function. *Diabetes* 57:967-979.

Granata R, Settanni F, Biancone L, Trovato L, Nano R, Bertuzzi F, Destefanis S, Annunziata M, Martinetti M, Catapano F, Ghe C, Isgaard J, Papotti M, Ghigo E, Muccioli G (2007) Acylated and unacylated ghrelin promote proliferation and inhibit apoptosis of pancreatic beta-cells and human islets: Involvement of 3',5'-cyclic adenosine monophosphate/protein kinase A, extracellular signal-regulated kinase 1/2, and phosphatidylinositol 3-Kinase/Akt signaling. *Endocrinology* 148:512-529.

Green BD, Irwin N, Flatt PR (2007) Direct and indirect effects of obestatin peptides on food intake and the regulation of glucose homeostasis and insulin secretion in mice. *Peptides* 28:981-987.

Gronberg M, Tsolakis AV, Magnusson L, Janson ET, Saras J (2008) Distribution of obestatin and ghrelin in human tissues: Immunoreactive cells in the gastrointestinal tract, pancreas, and mammary glands. *J Histochem Cytochem* 56:793-801.

Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, Smith RG, Van der Ploeg LH, Howard AD (1997) Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res Mol Brain Res* 48:23-29.

Guo ZF, Ren AJ, Zheng X, Qin YW, Cheng F, Zhang J, Wu H, Yuan WJ, Zou L (2008) Different responses of circulating ghrelin, obestatin levels to fasting, re-feeding and different food compositions, and their local expressions in rats. *Peptides* 29:1247-1254.

Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, Witcher DR, Luo S, Onyia JE, Hale JE (2008) Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci U S A* 105:6320-6325.

Han XF, Zhu YL, Hernandez M, Keating DJ, Chen C (2005) Ghrelin reduces voltage-gated potassium currents in GH3 cells via cyclic GMP pathways. *Endocrine* 28:217-224.

Hassouna R, Zizzari P, Tolle V (2010) The ghrelin/obestatin balance in the physiological and pathological control of GH secretion, body composition and food intake. *J Neuroendocrinol* .

Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB (2000) Isolation of INS-1-derived cell lines with robust ATP-sensitive K⁺ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* 49:424-430.

Holst B, Egerod KL, Schild E, Vickers SP, Cheetham S, Gerlach LO, Storjohann L, Stidsen CE, Jones R, Beck-Sickinger AG, Schwartz TW (2007) GPR39 signaling is stimulated by zinc ions but not by obestatin. *Endocrinology* 148:13-20.

Holst B, Egerod KL, Jin C, Petersen PS, Ostergaard MV, Hald J, Sprinkel AM, Stirling J, Mandrup-Poulsen T, Holst JJ, Thams P, Orskov C, Wierup N, Sundler F, Madsen OD, Schwartz TW (2009) G protein-coupled receptor 39 deficiency is associated with pancreatic islet dysfunction. *Endocrinology* 150:2577-2585.

Hoorens A, Van de Casteele M, Kloppel G, Pipeleers D (1996) Glucose promotes survival of rat pancreatic beta cells by activating synthesis of proteins which suppress a constitutive apoptotic program. *J Clin Invest* 98:1568-1574.

Hosoda H, Kojima M, Matsuo H, Kangawa K (2000) Ghrelin and des-acyl ghrelin: Two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochem Biophys Res Commun* 279:909-913.

Howard AD et al (1996) A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 273:974-977.

Hui H, Nourparvar A, Zhao X, Perfetti R (2003) Glucagon-like peptide-1 inhibits apoptosis of insulin-secreting cells via a cyclic 5'-adenosine monophosphate-dependent protein kinase A- and a phosphatidylinositol 3-kinase-dependent pathway. *Endocrinology* 144:1444-1455.

Irako T, Akamizu T, Hosoda H, Iwakura H, Ariyasu H, Tojo K, Tajima N, Kangawa K (2006) Ghrelin prevents development of diabetes at adult age in streptozotocin-treated newborn rats. *Diabetologia* 49:1264-1273.

Ishihara H, Asano T, Tsukuda K, Katagiri H, Inukai K, Anai M, Kikuchi M, Yazaki Y, Miyazaki JI, Oka Y (1993) Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia* 36:1139-1145.

Janicke RU, Sprengart ML, Wati MR, Porter AG (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem* 273:9357-9360.

Jetton TL, Lausier J, LaRock K, Trotman WE, Larmie B, Habibovic A, Peshavaria M, Leahy JL (2005) Mechanisms of compensatory beta-cell growth in insulin-resistant rats: Roles of akt kinase. *Diabetes* 54:2294-2304.

Jiang H, Betancourt L, Smith RG (2006) Ghrelin amplifies dopamine signaling by cross talk involving formation of growth hormone secretagogue receptor/dopamine receptor subtype 1 heterodimers. *Mol Endocrinol* 20:1772-1785.

Johansson I, Destefanis S, Aberg ND, Aberg MA, Blomgren K, Zhu C, Ghe C, Granata R, Ghigo E, Muccioli G, Eriksson PS, Isgaard J (2008) Proliferative and protective

effects of growth hormone secretagogues on adult rat hippocampal progenitor cells. *Endocrinology* 149:2191-2199.

Kargar C, Ktorza A (2008) Anatomical versus functional beta-cell mass in experimental diabetes. *Diabetes Obes Metab* 10 Suppl 4:43-53.

Kerem M, Salman B, Ozsoy S, Pasaoglu H, Bedirli A, Hazirolu R, Yilmaz TU (2008) Exogenous ghrelin enhances endocrine and exocrine regeneration in pancreatectomized rats. *J Gastrointest Surg* .

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26:239-257.

Kineman RD, Luque RM (2007) Evidence that ghrelin is as potent as growth hormone (GH)-releasing hormone (GHRH) in releasing GH from primary pituitary cell cultures of a nonhuman primate (*papio anubis*), acting through intracellular signaling pathways distinct from GHRH. *Endocrinology* 148:4440-4449.

Kinzig KP, Hargrave SL, Tao EE (2009) Central and peripheral effects of chronic food restriction and weight restoration in the rat. *Am J Physiol Endocrinol Metab* 296:E282-90.

Kirchner H, Gutierrez JA, Solenberg PJ, Pfluger PT, Czyzyk TA, Willency JA, Schurmann A, Joost HG, Jandacek RJ, Hale JE, Heiman ML, Tschop MH (2009) GOAT links dietary lipids with the endocrine control of energy balance. *Nat Med* 15:741-745.

Kobelt P, Wisser AS, Stengel A, Goebel M, Bannert N, Gourcerol G, Inhoff T, Noetzel S, Wiedenmann B, Klapp BF, Tache Y, Monnikes H (2008a) Peripheral obestatin has no effect on feeding behavior and brain fos expression in rodents. *Peptides* 29:1018-1027.

Kobelt P, Wisser AS, Stengel A, Goebel M, Inhoff T, Noetzel S, Veh RW, Bannert N, van der Voort I, Wiedenmann B, Klapp BF, Tache Y, Monnikes H (2008b) Peripheral injection of ghrelin induces fos expression in the dorsomedial hypothalamic nucleus in rats. *Brain Res* 1204:77-86.

Kohno D, Sone H, Minokoshi Y, Yada T (2008) Ghrelin raises $[Ca^{2+}]_i$ via AMPK in hypothalamic arcuate nucleus NPY neurons. *Biochem Biophys Res Commun* 366:388-392.

Kohno D, Gao HZ, Muroya S, Kikuyama S, Yada T (2003) Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca^{2+} signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes* 52:948-956.

- Kojima M, Kangawa K (2008) Structure and function of ghrelin. *Results Probl Cell Differ* 46:89-115.
- Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402:656-660.
- Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, Burgering BM (1999) Direct control of the forkhead transcription factor AFX by protein kinase B. *Nature* 398:630-634.
- Korbonits M, Goldstone AP, Gueorguiev M, Grossman AB (2004) Ghrelin--a hormone with multiple functions. *Front Neuroendocrinol* 25:27-68.
- Koshkin V, Dai FF, Robson-Doucette CA, Chan CB, Wheeler MB (2008) Limited mitochondrial permeabilization is an early manifestation of palmitate-induced lipotoxicity in pancreatic beta-cells. *J Biol Chem* 283:7936-7948.
- Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Kohts K, Kwiatkowski DJ, Williams LT (1997) Caspase-3-generated fragment of gelsolin: Effector of morphological change in apoptosis. *Science* 278:294-298.
- Lai E, Bikopoulos G, Wheeler MB, Rozakis-Adcock M, Volchuk A (2008) Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic beta-cells. *Am J Physiol Endocrinol Metab* 294:E540-50.
- Lauwers E, Landuyt B, Arckens L, Schoofs L, Luyten W (2006) Obestatin does not activate orphan G protein-coupled receptor GPR39. *Biochem Biophys Res Commun* 351:21-25.
- Lear PV, Iglesias MJ, Feijoo-Bandin S, Rodriguez-Penas D, Mosquera-Leal A, Garcia-Rua V, Gualillo O, Ghe C, Arnoletti E, Muccioli G, Dieguez C, Gonzalez-Juanatey JR, Lago F (2010) Des-acyl ghrelin has specific binding sites and different metabolic effects from ghrelin in cardiomyocytes. *Endocrinology* .
- Lee HM, Wang G, Englander EW, Kojima M, Greeley GH, Jr (2002) Ghrelin, a new gastrointestinal endocrine peptide that stimulates insulin secretion: Enteric distribution, ontogeny, influence of endocrine, and dietary manipulations. *Endocrinology* 143:185-190.
- Li L, El-Kholy W, Rhodes CJ, Brubaker PL (2005) Glucagon-like peptide-1 protects beta cells from cytokine-induced apoptosis and necrosis: Role of protein kinase B. *Diabetologia* 48:1339-1349.
- Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91:479-489.

- Lingohr MK, Buettner R, Rhodes CJ (2002) Pancreatic beta-cell growth and survival--a role in obesity-linked type 2 diabetes? *Trends Mol Med* 8:375-384.
- Liu W, Chin-Chance C, Lee EJ, Lowe WL, Jr (2002) Activation of phosphatidylinositol 3-kinase contributes to insulin-like growth factor I-mediated inhibition of pancreatic beta-cell death. *Endocrinology* 143:3802-3812.
- Longo KA, Charoenthongtrakul S, Giuliana DJ, Govek EK, McDonagh T, Qi Y, DiStefano PS, Geddes BJ (2008) Improved insulin sensitivity and metabolic flexibility in ghrelin receptor knockout mice. *Regul Pept* 150:55-61.
- MacDonald PE, Joseph JW, Rorsman P (2005) Glucose-sensing mechanisms in pancreatic beta-cells. *Philos Trans R Soc Lond B Biol Sci* 360:2211-2225.
- Maechler P, Carobbio S, Rubi B (2006) In beta-cells, mitochondria integrate and generate metabolic signals controlling insulin secretion. *Int J Biochem Cell Biol* 38:696-709.
- Maestre I, Jordan J, Calvo S, Reig JA, Cena V, Soria B, Prentki M, Roche E (2003) Mitochondrial dysfunction is involved in apoptosis induced by serum withdrawal and fatty acids in the beta-cell line INS-1. *Endocrinology* 144:335-345.
- Malagon MM, Luque RM, Ruiz-Guerrero E, Rodriguez-Pacheco F, Garcia-Navarro S, Casanueva FF, Gracia-Navarro F, Castano JP (2003) Intracellular signaling mechanisms mediating ghrelin-stimulated growth hormone release in somatotropes. *Endocrinology* 144:5372-5380.
- Martinez SC, Cras-Meneur C, Bernal-Mizrachi E, Permutt MA (2006) Glucose regulates Foxo1 through insulin receptor signaling in the pancreatic islet beta-cell. *Diabetes* 55:1581-1591.
- Mayorov AV, Amara N, Chang JY, Moss JA, Hixon MS, Ruiz DI, Meijler MM, Zorrilla EP, Janda KD (2008) Catalytic antibody degradation of ghrelin increases whole-body metabolic rate and reduces refeeding in fasting mice. *Proc Natl Acad Sci U S A* 105:17487-17492.
- McKee KK, Palyha OC, Feighner SD, Hreniuk DL, Tan CP, Phillips MS, Smith RG, Van der Ploeg LH, Howard AD (1997) Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors. *Mol Endocrinol* 11:415-423.
- Meier JJ, Butler AE, Saisho Y, Monchamp T, Galasso R, Bhushan A, Rizza RA, Butler PC (2008) Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. *Diabetes* 57:1584-1594.
- Moechars D, Depoortere I, Moreaux B, de Smet B, Goris I, Hoskens L, Daneels G, Kass S, Ver Donck L, Peeters T, Coulie B (2006) Altered gastrointestinal and metabolic

function in the GPR39-obestatin receptor-knockout mouse. *Gastroenterology* 131:1131-1141.

Mondal MS, Date Y, Yamaguchi H, Toshinai K, Tsuruta T, Kangawa K, Nakazato M (2005) Identification of ghrelin and its receptor in neurons of the rat arcuate nucleus. *Regul Pept* 126:55-59.

Montanya E, Nacher V, Biarnes M, Soler J (2000) Linear correlation between beta-cell mass and body weight throughout the lifespan in lewis rats: Role of beta-cell hyperplasia and hypertrophy. *Diabetes* 49:1341-1346.

Morash MG, Gagnon J, Nelson S, Anini Y (2010) Tissue distribution and effects of fasting and obesity on the ghrelin axis in mice. *Regul Pept* .

Morishima N, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y (2002) An endoplasmic reticulum stress-specific caspase cascade in apoptosis. cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem* 277:34287-34294.

Movassat J, Saulnier C, Serradas P, Portha B (1997) Impaired development of pancreatic beta-cell mass is a primary event during the progression to diabetes in the GK rat. *Diabetologia* 40:916-925.

Muccioli G, Baragli A, Granata R, Papotti M, Ghigo E (2007) Heterogeneity of ghrelin/growth hormone secretagogue receptors. toward the understanding of the molecular identity of novel ghrelin/GHS receptors. *Neuroendocrinology* 86:147-164.

Muharram G, Beucher A, Moerman E, Belaich S, Gmyr V, Vandewalle B, Pattou F, Kerr-Conte J (2005) Endocrine pancreatic tissue plasticity in obese humans is associated with cytoplasmic expression of PBX-1 in pancreatic ductal cells. *Biochem Biophys Res Commun* 333:1153-1159.

Musmann R, Geese M, Harder F, Kegel S, Andag U, Lomow A, Burk U, Onichtchouk D, Dohrmann C, Austen M (2007) Inhibition of GSK3 promotes replication and survival of pancreatic beta cells. *J Biol Chem* 282:12030-12037.

Nakahara K, Nakagawa M, Baba Y, Sato M, Toshinai K, Date Y, Nakazato M, Kojima M, Miyazato M, Kaiya H, Hosoda H, Kangawa K, Murakami N (2006) Maternal ghrelin plays an important role in rat fetal development during pregnancy. *Endocrinology* 147:1333-1342.

Nakashima K, Kanda Y, Hirokawa Y, Kawasaki F, Matsuki M, Kaku K (2008) MIN6 is not a pure beta cell line but a mixed cell line with other pancreatic endocrine hormones. *Endocr J* .

Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S (2001) A role for ghrelin in the central regulation of feeding. *Nature* 409:194-198.

Nanzer AM, Khalaf S, Mozid AM, Fowkes RC, Patel MV, Burrin JM, Grossman AB, Korbonits M (2004) Ghrelin exerts a proliferative effect on a rat pituitary somatotroph cell line via the mitogen-activated protein kinase pathway. *Eur J Endocrinol* 151:233-240.

Navarro-Tableros V, Sanchez-Soto MC, Garcia S, Hiriart M (2004) Autocrine regulation of single pancreatic beta-cell survival. *Diabetes* 53:2018-2023.

Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376:37-43.

Ohsugi M, Cras-Meneur C, Zhou Y, Bernal-Mizrachi E, Johnson JD, Luciani DS, Polonsky KS, Permutt MA (2005) Reduced expression of the insulin receptor in mouse insulinoma (MIN6) cells reveals multiple roles of insulin signaling in gene expression, proliferation, insulin content, and secretion. *J Biol Chem* 280:4992-5003.

Orecna M, Hafko R, Bacova Z, Podskocova J, Chorvat D, Jr, Strbak V (2008) Different secretory response of pancreatic islets and insulin secreting cell lines INS-1 and INS-1E to osmotic stimuli. *Physiol Res* 57:935-945.

Otto B, Cuntz U, Fruehauf E, Wawarta R, Folwaczny C, Riepl RL, Heiman ML, Lehnert P, Fichter M, Tschop M (2001) Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. *Eur J Endocrinol* 145:669-673.

Parsons JA, Brelje TC, Sorenson RL (1992) Adaptation of islets of langerhans to pregnancy: Increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. *Endocrinology* 130:1459-1466.

Pfluger PT, Kirchner H, Gunnel S, Schrott B, Perez-Tilve D, Fu S, Benoit SC, Horvath T, Joost HG, Wortley KE, Sleeman MW, Tschop MH (2008) Simultaneous deletion of ghrelin and its receptor increases motor activity and energy expenditure. *Am J Physiol Gastrointest Liver Physiol* 294:G610-8.

Pick A, Clark J, Kubstrup C, Levisetti M, Pugh W, Bonner-Weir S, Polonsky KS (1998) Role of apoptosis in failure of beta-cell mass compensation for insulin resistance and beta-cell defects in the male Zucker diabetic fatty rat. *Diabetes* 47:358-364.

Porter AG, Janicke RU (1999) Emerging roles of caspase-3 in apoptosis. *Cell Death Differ* 6:99-104.

Poykko SM, Kellokoski E, Horkko S, Kauma H, Kesaniemi YA, Ukkola O (2003) Low plasma ghrelin is associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes. *Diabetes* 52:2546-2553.

- Qader SS, Hakanson R, Rehfeld JF, Lundquist I, Salehi A (2008) Proghrelin-derived peptides influence the secretion of insulin, glucagon, pancreatic polypeptide and somatostatin: A study on isolated islets from mouse and rat pancreas. *Regul Pept* 146:230-237.
- Qader SS, Lundquist I, Ekelund M, Hakanson R, Salehi A (2005) Ghrelin activates neuronal constitutive nitric oxide synthase in pancreatic islet cells while inhibiting insulin release and stimulating glucagon release. *Regul Pept* 128:51-56.
- Rachdi L, Balcazar N, Osorio-Duque F, Elghazi L, Weiss A, Gould A, Chang-Chen KJ, Gambello MJ, Bernal-Mizrachi E (2008) Disruption of Tsc2 in pancreatic beta cells induces beta cell mass expansion and improved glucose tolerance in a TORC1-dependent manner. *Proc Natl Acad Sci U S A* 105:9250-9255.
- Reimer MK, Pacini G, Ahren B (2003) Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. *Endocrinology* 144:916-921.
- Ren AJ, Guo ZF, Wang YK, Wang LG, Wang WZ, Lin L, Zheng X, Yuan WJ (2008) Inhibitory effect of obestatin on glucose-induced insulin secretion in rats. *Biochem Biophys Res Commun* 369:969-972.
- Rhodes CJ (2005) Type 2 diabetes-a matter of beta-cell life and death? *Science* 307:380-384.
- Rodriguez-Pacheco F, Luque RM, Tena-Sempere M, Malagon MM, Castano JP (2008) Ghrelin induces growth hormone secretion via a nitric oxide/cGMP signalling pathway. *J Neuroendocrinol* 20:406-412.
- Rodriguez-Pacheco F, Luque RM, Garcia-Navarro S, Gracia-Navarro F, Castano JP, Malagon MM (2005) Ghrelin induces growth hormone (GH) secretion via nitric oxide (NO)/cGMP signaling. *Ann N Y Acad Sci* 1040:452-453.
- Salehi A, Dornonville de la Cour C, Hakanson R, Lundquist I (2004) Effects of ghrelin on insulin and glucagon secretion: A study of isolated pancreatic islets and intact mice. *Regul Pept* 118:143-150.
- Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307:1098-1101.
- Sato M, Nakahara K, Goto S, Kaiya H, Miyazato M, Date Y, Nakazato M, Kangawa K, Murakami N (2006) Effects of ghrelin and des-acyl ghrelin on neurogenesis of the rat fetal spinal cord. *Biochem Biophys Res Commun* 350:598-603.
- Sato T, Fukue Y, Teranishi H, Yoshida Y, Kojima M (2005) Molecular forms of hypothalamic ghrelin and its regulation by fasting and 2-deoxy-d-glucose administration. *Endocrinology* 146:2510-2516.

- Scaglia L, Cahill CJ, Finegood DT, Bonner-Weir S (1997) Apoptosis participates in the remodeling of the endocrine pancreas in the neonatal rat. *Endocrinology* 138:1736-1741.
- Shearman LP, Wang SP, Helmling S, Stribling DS, Mazur P, Ge L, Wang L, Klussmann S, Macintyre DE, Howard AD, Strack AM (2006) Ghrelin neutralization by a ribonucleic acid-SPM ameliorates obesity in diet-induced obese mice. *Endocrinology* 147:1517-1526.
- Shuto Y, Shibasaki T, Otagiri A, Kuriyama H, Ohata H, Tamura H, Kamegai J, Sugihara H, Oikawa S, Wakabayashi I (2002) Hypothalamic growth hormone secretagogue receptor regulates growth hormone secretion, feeding, and adiposity. *J Clin Invest* 109:1429-1436.
- Soares JB, Leite-Moreira AF (2008) Ghrelin, des-acyl ghrelin and obestatin: Three pieces of the same puzzle. *Peptides* 29:1255-1270.
- Sone H, Kagawa Y (2005) Pancreatic beta cell senescence contributes to the pathogenesis of type 2 diabetes in high-fat diet-induced diabetic mice. *Diabetologia* 48:58-67.
- Srinivasan S, Bernal-Mizrachi E, Ohsugi M, Permutt MA (2002) Glucose promotes pancreatic islet beta-cell survival through a PI 3-kinase/Akt-signaling pathway. *Am J Physiol Endocrinol Metab* 283:E784-93.
- Strassburg S, Anker SD, Castaneda TR, Burget L, Perez-Tilve D, Pfluger PT, Nogueiras R, Halem H, Dong JZ, Culler MD, Datta R, Tschop MH (2008) Long-term effects of ghrelin and ghrelin receptor agonists on energy balance in rats. *Am J Physiol Endocrinol Metab* 295:E78-84.
- Sun Y, Ahmed S, Smith RG (2003) Deletion of ghrelin impairs neither growth nor appetite. *Mol Cell Biol* 23:7973-7981.
- Sun Y, Butte NF, Garcia JM, Smith RG (2008) Characterization of adult ghrelin and ghrelin receptor knockout mice under positive and negative energy balance. *Endocrinology* 149:843-850.
- Sun Y, Wang P, Zheng H, Smith RG (2004) Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. *Proc Natl Acad Sci U S A* 101:4679-4684.
- Sun Y, Asnicar M, Saha PK, Chan L, Smith RG (2006) Ablation of ghrelin improves the diabetic but not obese phenotype of ob/ob mice. *Cell Metab* 3:379-386.
- Szabadkai G, Duchon MR (2009) Mitochondria mediated cell death in diabetes. *Apoptosis* 14:1405-1423.

- Takahashi T, Ida T, Sato T, Nakashima Y, Nakamura Y, Tsuji A, Kojima M (2009) Production of n-octanoyl-modified ghrelin in cultured cells requires prohormone processing protease and ghrelin O-acyltransferase, as well as n-octanoic acid. *J Biochem* 146:675-682.
- Tang SQ, Jiang QY, Zhang YL, Zhu XT, Shu G, Gao P, Feng DY, Wang XQ, Dong XY (2008) Obestatin: Its physicochemical characteristics and physiological functions. *Peptides* 29:639-645.
- Theander-Carrillo C, Wiedmer P, Cettour-Rose P, Nogueiras R, Perez-Tilve D, Pfluger P, Castaneda TR, Muzzin P, Schurmann A, Szanto I, Tschop MH, Rohner-Jeanrenaud F (2006) Ghrelin action in the brain controls adipocyte metabolism. *J Clin Invest* 116:1983-1993.
- Thompson NM, Gill DA, Davies R, Loveridge N, Houston PA, Robinson IC, Wells T (2004) Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretagogue receptor. *Endocrinology* 145:234-242.
- Toshinai K, Yamaguchi H, Sun Y, Smith RG, Yamanaka A, Sakurai T, Date Y, Mondal MS, Shimbara T, Kawagoe T, Murakami N, Miyazato M, Kangawa K, Nakazato M (2006) Des-acyl ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor. *Endocrinology* 147:2306-2314.
- Tremblay F, Perreault M, Klaman LD, Tobin JF, Smith E, Gimeno RE (2007) Normal food intake and body weight in mice lacking the G protein-coupled receptor GPR39. *Endocrinology* 148:501-506.
- Tremblay F, Richard AM, Will S, Syed J, Stedman N, Perreault M, Gimeno RE (2009) Disruption of G protein-coupled receptor 39 impairs insulin secretion in vivo. *Endocrinology* 150:2586-2595.
- Tschop M, Smiley DL, Heiman ML (2000) Ghrelin induces adiposity in rodents. *Nature* 407:908-913.
- Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML (2001) Circulating ghrelin levels are decreased in human obesity. *Diabetes* 50:707-709.
- Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furth EE, Polonsky KS, Naji A, Birnbaum MJ (2001) Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. *Nat Med* 7:1133-1137.
- Unniappan S, Speck M, Kieffer TJ (2008) Metabolic effects of chronic obestatin infusion in rats. *Peptides* 29:1354-1361.

Volante M, Allia E, Gugliotta P, Funaro A, Broglio F, Deghenghi R, Muccioli G, Ghigo E, Papotti M (2002) Expression of ghrelin and of the GH secretagogue receptor by pancreatic islet cells and related endocrine tumors. *J Clin Endocrinol Metab* 87:1300-1308.

Walia P, Asadi A, Kieffer TJ, Johnson JD, Chanoine JP (2008) Ontogeny of ghrelin, obestatin, preproghrelin, and prohormone convertases in rat pancreas and stomach. *Pediatr Res* .

Wang Q, Brubaker PL (2002) Glucagon-like peptide-1 treatment delays the onset of diabetes in 8 week-old db/db mice. *Diabetologia* 45:1263-1273.

Wang Q, Li L, Xu E, Wong V, Rhodes C, Brubaker PL (2004) Glucagon-like peptide-1 regulates proliferation and apoptosis via activation of protein kinase B in pancreatic INS-1 beta cells. *Diabetologia* 47:478-487.

Wang W, Zhang D, Zhao H, Chen Y, Liu Y, Cao C, Han L, Liu G (2010a) Ghrelin inhibits cell apoptosis induced by lipotoxicity in pancreatic beta-cell line. *Regul Pept* 161:43-50.

Wang W, Liu Y, Chen Y, Cao C, Xiang Y, Zhang D, Han L, Zhao H, Liu G (2010b) Inhibition of Foxo1 mediates protective effects of ghrelin against lipotoxicity in MIN6 pancreatic beta-cells. *Peptides* 31:307-314.

Wang Y, Nishi M, Doi A, Shono T, Furukawa Y, Shimada T, Furuta H, Sasaki H, Nanjo K (2010c) Ghrelin inhibits insulin secretion through the AMPK-UCP2 pathway in beta cells. *FEBS Lett* .

Weir GC, Bonner-Weir S (2004) Five stages of evolving beta-cell dysfunction during progression to diabetes. *Diabetes* 53 Suppl 3:S16-21.

Weir GC, Laybutt DR, Kaneto H, Bonner-Weir S, Sharma A (2001) Beta-cell adaptation and decompensation during the progression of diabetes. *Diabetes* 50 Suppl 1:S154-9.

Wen LP, Fahrni JA, Troie S, Guan JL, Orth K, Rosen GD (1997) Cleavage of focal adhesion kinase by caspases during apoptosis. *J Biol Chem* 272:26056-26061.

Widlak P (2000) The DFF40/CAD endonuclease and its role in apoptosis. *Acta Biochim Pol* 47:1037-1044.

Wierup N, Sundler F (2005) Ultrastructure of islet ghrelin cells in the human fetus. *Cell Tissue Res* 319:423-428.

Wierup N, Svensson H, Mulder H, Sundler F (2002) The ghrelin cell: A novel developmentally regulated islet cell in the human pancreas. *Regul Pept* 107:63-69.

- Wierup N, Yang S, McEvelly RJ, Mulder H, Sundler F (2004) Ghrelin is expressed in a novel endocrine cell type in developing rat islets and inhibits insulin secretion from INS-1 (832/13) cells. *J Histochem Cytochem* 52:301-310.
- Wortley KE, del Rincon JP, Murray JD, Garcia K, Iida K, Thorner MO, Sleeman MW (2005) Absence of ghrelin protects against early-onset obesity. *J Clin Invest* 115:3573-3578.
- Wrede CE, Dickson LM, Lingohr MK, Briaud I, Rhodes CJ (2002) Protein kinase B/Akt prevents fatty acid-induced apoptosis in pancreatic beta-cells (INS-1). *J Biol Chem* 277:49676-49684.
- Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA, Bloom SR (2001a) Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab* 86:5992.
- Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA, Bloom SR (2001b) Ghrelin causes hyperphagia and obesity in rats. *Diabetes* 50:2540-2547.
- Yang H, Youm YH, Nakata C, Dixit VD (2007) Chronic caloric restriction induces forestomach hypertrophy with enhanced ghrelin levels during aging. *Peptides* 28:1931-1936.
- Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL (2008) Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* 132:387-396.
- Yoon KH, Ko SH, Cho JH, Lee JM, Ahn YB, Song KH, Yoo SJ, Kang MI, Cha BY, Lee KW, Son HY, Kang SK, Kim HS, Lee IK, Bonner-Weir S (2003) Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea. *J Clin Endocrinol Metab* 88:2300-2308.
- Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, Hsueh AJ (2005) Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* 310:996-999.
- Zhang JV, Jahr H, Luo CW, Klein C, Van Kolen K, Ver Donck L, De A, Baart E, Li J, Moechars D, Hsueh AJ (2008a) Obestatin induction of early-response gene expression in gastrointestinal and adipose tissues and the mediatory role of G protein-coupled receptor, GPR39. *Mol Endocrinol* 22:1464-1475.
- Zhang W, Chai B, Li JY, Wang H, Mulholland MW (2008b) Effect of des-acyl ghrelin on adiposity and glucose metabolism. *Endocrinology* 149:4710-4716.

Zhang Y, Ying B, Shi L, Fan H, Yang D, Xu D, Wei Y, Hu X, Zhang Y, Zhang X, Wang T, Liu D, Dou L, Chen G, Jiang F, Wen F (2007) Ghrelin inhibit cell apoptosis in pancreatic beta cell line HIT-T15 via mitogen-activated protein kinase/phosphoinositide 3-kinase pathways. *Toxicology* 237:194-202.

Zhu X, Cao Y, Voogd K, Steiner DF (2006) On the processing of proghrelin to ghrelin. *J Biol Chem* 281:38867-38870.

Zigman JM, Nakano Y, Coppari R, Balthasar N, Marcus JN, Lee CE, Jones JE, Deysher AE, Waxman AR, White RD, Williams TD, Lachey JL, Seeley RJ, Lowell BB, Elmquist JK (2005) Mice lacking ghrelin receptors resist the development of diet-induced obesity. *J Clin Invest* 115:3564-3572.

Zorrilla EP, Iwasaki S, Moss JA, Chang J, Otsuji J, Inoue K, Meijler MM, Janda KD (2006) Vaccination against weight gain. *Proc Natl Acad Sci U S A* 103:13226-13231.