POMC Is Expressed in Pancreatic Alpha Cells

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POMC IS EXPRESSED IN PANCREATIC ALPHA CELLS

A Thesis
Presented to
the Faculty of Natural Sciences and Mathematics
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of the Requirements for the Degree
Master of Science

by
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Abstract

Pancreatic alpha cells secrete glucagon to increase blood glucose during hypoglycemia. Currently, the mechanisms that initiate glucagon secretion are not well understood. This study investigates the expression of pro-opiomelanocortin (POMC), a potent regulator of glucagon secretion, in alpha cells.

Insulin tolerance tests in mice lacking the POMC gene showed a correlation between α-MSH, and glucagon secretion from alpha cells (Hochgeschwender et al. 2003). Results from a previous study showed that the POMC product, melanocortin α-MSH, acts through a melanocortin receptor 4 (MC4R) at the surface of the alpha cells to trigger the release of glucagon [Angleson, unpublished data, Lumsden MS thesis 2008]. The results of the current study indicate that POMC is expressed in pancreatic alpha cells.

Analysis through quantitative PCR, Western blot, as well as a combination of fluorescent techniques was used to investigate the expression of POMC in mouse pancreatic tissue and dispersed islets cells, as well as an alpha cell line. POMC was found to be expressed in alpha cells, not in beta cells of the pancreas. The results suggest a mechanism for positive autocrine feedback of alpha cells.
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Mechanism of Insulin secretion

Diabetes mellitus is characterized by hyperglycemia due to insulin deficiency. This deficiency is caused by pancreatic beta cell destruction or disturbed secretion of the hormone. Insulin resistance is a main cause of diabetes mellitus in obese patients (Hochgeschwender et al. 2003).

Islet beta cells secrete insulin when blood glucose is high. Insulin acts mainly on muscle, liver and adipose tissue with an anabolic effect inducing the corporation of glucose into these tissues and its accumulation as glycogen and fat. Regulated insulin secretion is relatively well understood.

Glucose enters beta cells mainly via the Glu-2 transporter. Metabolism of glucose is essential for stimulation of insulin release. Metabolism of glucose leads to an increase in ATP/ADP ratio and closure of ATP sensitive potassium channels; the closure of these channels leads to depolarization of beta cells and the opening of voltage-gated calcium channels (L-type calcium channel) leading to an increase in the cytosolic calcium.
concentration that triggers exocytosis. P/Q calcium channels also play a crucial role in the exocytosis process (Gisela Drew et al. 2010). cAMP promotes exocytosis by facilitating the generation of calcium signals through sensitization of the secretary machinery to calcium. The phosphorylation of L-type calcium channel by cAMP–dependent kinase enhance the open probability of the channel as well as stimulate mobilization of granules via protein kinase A and Epac pathway (Seth et al. 2007).

Figure1: Mechanism of insulin secretion
Mechanism of glucagon secretion

The secretion of glucagon by pancreatic alpha cells plays a critical role in the regulation of glycaemia. This hormone counteracts hypoglycemia and opposes insulin actions by stimulating hepatic glucose synthesis and mobilization thereby increasing the blood glucose concentration (Ivan et al. 2008). Failure of glucose to suppress the release of glucose-elevating glucagon aggravates hyperglycemia in diabetic patients. Further glucose elevation has even been found to stimulate glucagon secretion (Vieira et al. 2006).

The regulation of glucagon secretion remains hotly debated. The control of glucagon secretion from alpha cells involves paracrine [mediated by the factors released by pancreatic beta and delta cells], neuronal [mediated by the autonomic nervous system], and intrinsic mechanisms [within the alpha cell itself]. All of these mechanisms potentially contribute to the physiological regulation of glucagon secretion. However, the
observation that glucose remains capable of regulating glucagon release in isolated pancreatic islets, where the nerves have been severed, demonstrates the existence of non-neuronal regulatory mechanisms exerted within the islet itself (Rorsman et al. 2008).
Paracrine regulation of glucagon secretion

The significance of paracrine signaling has been highlighted by the recent observation that high blood glucose stimulates rather than inhibits glucagon release in isolated rat alpha cells, i.e. when the paracrine control has been disrupted (Ishihara et al. 2003). The paradoxical stimulation of glucagon secretion by glucose in isolated alpha cells is counteracted by introduction of paracrine regulators such as insulin, zinc, GABA, and somatostatin. All of these factors directly inhibit alpha cells. Glucagon secretion from alpha cells is high in absence of glucose and suppressed by increasing the glucose concentration. Close to maximal inhibition is observed at 3mM glucose, a concentration not associated with any marked stimulation of insulin or somatostatin release (Vieira et al. 2005). In addition to the inhibitory effect seen at 3-10 mM glucose, glucose also exerts a stimulatory effect on glucagon secretion at 20 mM glucose. The finding that inhibition of glucagon secretion occurs at lower concentrations than those required to initiate insulin and somatostatin release makes it unlikely that this suppression depends entirely on factors secreted by beta cells (Rorsman et al. 2008).
Intrinsic alpha cells regulation of glucagon secretion

Alpha cells possess an intrinsic glucose-sensing mechanism that accounts for their ability to respond to glucose at concentrations too low to elicit insulin or somatostatin secretion. Some studies have shown that the low glucose levels are not a direct stimulator of glucagon secretion (Gromada et al. 2004, Gustavson et al. 2003). However, other studies indicate a drop in glucose is sufficient to trigger calcium influx and glucagon secretion (Rorsman et al. 2011).

The nature of this intrinsic regulation remain poorly defined, but several studies suggest that the intrinsic glucose sensing of alpha cells involves ATP-sensitive potassium channels similar to those found in beta cells (Bokvis et al. 2009, Gromada et al. 2004). Both alpha and beta cell K<sub>ATP</sub> channels have the same molecular composition [sulfonylurea receptor SUR1, and inward rectifier potassium channels Kir6.2]. Interestingly, K<sub>ATP</sub> channels in the alpha cells are more sensitive to ATP inhibition than in those beta cells. At low glucose levels, the activity of K<sub>ATP</sub> channels hyperpolarizes the membrane potential to about –60 mV. At this voltage, T-type calcium channels open which depolarize the membrane potential to a level where sodium channels are activated leading to regenerative action potentials (Gromada et al. 2004, Macdonald et al. 2007).
Depolarization leads to opening of L-type calcium channels and N-type calcium channels, which trigger the exocytosis of glucagon-containing secretory granules. Conversely, an increase in extracellular glucose levels raises the cytosolic ATP/ADP ratio and blocks K\textsubscript{ATP} channels. The alpha cells then depolarize to a membrane potential range where the sodium and calcium channels become inactivated (Gromada et al. 2004, Macdonald et al. 2007). As a consequence, electrical activity, calcium signals and glucagon release are inhibited. Thus, glucagon secretion from alpha cells is mainly supported by intermediate K\textsubscript{ATP} channel activity that maintains a membrane potential range able to sustain regenerative electrical activity (MacDonald et al. 2007).

Nevertheless, this scheme has been argued by studies demonstrating that glucose hyperpolarizes rather than depolarizes alpha cells (Liu et al. 2004). In addition, it has been proposed that glucose inhibits glucagon release by decreasing a depolarizing calcium store-operated current resulting in depolarization independent of K\textsubscript{ATP} channels (Liu et al. 2004, Vieira et al. 2007).

Homozygous POMC mutants were unable to recover from insulin-induced hypoglycemia due to a defect in the glucagon-mediated counterregulatory response (Hochgeschwender et al. 2003). Both peripheral administration of a αMSH analog and supplementation with corticosterone alleviated the hypoglycemia after insulin challenge. However, when injected with ACTH alone, the phenotype was not rescued. This study
shows that there is a correlation between melanocortin alpha MSH and glucagon secretion in alpha cells. Another study found that a POMC product, α MSH, is acting through a melanocortin receptor at the surface of alpha cells to trigger the release of glucagon (Angleson, Lumsden, MS thesis 2008). The receptor MC4 was found to be expressed in whole mouse islets and as well in alpha cells. However, MC3 does not appear in islets or alpha cells. This study concluded that alpha MSH acts through MC4Rs to trigger glucagon secretion in alpha cells (Angleson, Lumsden, MS thesis 2008).
Figure 2: Glucose regulation of the mouse pancreatic alpha cell. A: At low glucose, KATP channel activity is low. B: At high glucose level KATP channels close completely (Gromada.2004).
POMC

Proopiomelanocortin is a 31 kDa precursor protein that is the source of many biologically active peptides. The protein is known to be synthesized within the corticotroph cells of the anterior pituitary, the melanotroph in the intermediate pituitary, and some neurons in the CNS (Strand et al. 1999). POMC undergoes extensive processing in a tissue specific manner through cleavage by prohormone convertase enzymes. There are eight possible cleavage sites which can yield as many as ten active peptides, although in the anterior pituitary only four of these sites are used. The result is two major end products (generated by PC 1/3), which are the adrenocorticotrophin hormone [ACTH] and lipotropin beta hormone [β-LPH]. Separate cleavage takes place in the intermediate lobe of the anterior pituitary yielding peptides such as corticotrophin-like intermediate peptide [CLIP], gamma–lipotropin, α-MSH and beta endorphin (Strand et al. 1999) [Figure 3]. POMC derived peptide α-MSH has been shown to act on alpha cells, contributing to the counterregulatory response during insulin tolerance tests (Hochgeschwender et al. 2003).

The melanocortin α-MSH is derived from the POMC gene; however, the origin of this melanocortin that regulates alpha cell function is unknown. Some possibilities as to the
origin of $\alpha$-MSH include blood circulation, and paracrine or autocrine tissues within islets. If $\alpha$-MSH is arriving at islets via blood circulation, it would likely indicate that $\alpha$-MSH is being secreted from the anterior pituitary. However, ACTH is secreted from the anterior pituitary, and $\alpha$-MSH secreted from the intermediate lobe not from anterior pituitary (Reisine et al. 1983, Strand et al. 1999). Another site of POMC expression is the CNS, although CNS, neurons are not likely directly involved in the $\alpha$-MSH stimulation of alpha cells due to the blood-brain barrier. Pancreatic islets are innervated by neurons which could suggest other possibilities regarding the origin of $\alpha$-MSH (Razavi et al. 2006). Yet, it is possible that $\alpha$-MSH is released from islet cells as paracrine or autocrine signal. PCR analysis in a previous study (Angleson, Lumsden, MS thesis 2008) showed POMC signal in islets as well as in an alpha cell clonal cell line, $\alpha$-TC1-6 cells. The goal of this study was to determine the localization of POMC as a precursor to the melanocortin that regulates alpha cell secretion. The results indicate that the pancreatic alpha cells themselves express POMC.
Figure 3: POMC processing of α-MSH
MATERIAL AND METHODS

Glucagon Secretion and \(\alpha\)-TC1-6 Cells as a Model

The current study utilized the alpha cell line \(\alpha\)-TC1-6, a mouse islet-cell line transformed by a large simian virus tumor (T) antigen. This cell line is a commonly used model of the pancreatic alpha cell, and through many studies have been shown to be a reliable cell line for hormone secretion studies (Rouillé et al. 1994). Above all, they are a good model for examining alpha cell gene expression, because, unlike other glucagon-secreting cell lines, they are homogeneous, do not express the hormones insulin or somatostatin, and respond to hypoglycemia.

AtT-20 pituitary cell line

In the current study, the AtT-20 cell line was used as a control. AtT-20 cells are a neuroendocrine line derived from ACTH-secreting adenocarcinoma in the anterior pituitary (Schiller et al. 2000). The AtT-20 cell line is used as a model to study the neuropeptide secretion and glucocorticoid regulation.
Cell Culture

Both cell lines used for the current experiments were maintained in culture. αTC1-6 cells were cultured in DMEM with glucose adjusted to 16mM and 10% heat-inactivated FBS, supplemented with 1mM Na-pyruvate, and MEM non-essential amino acids. AtT-20 cells were maintained in DMEM/F12, FBS, .06% glucose, HEPES and pH was adjusted to 7.2. Islets isolated from normal C57BL6 mice were maintained in HBSS/HEPES 0.01%, BSA 0.1%, PSN, DNAase solution.

Immunofluorescence and Histology

Protein expression was determined by immunofluorescence on sections of paraffin-embedded mouse pancreatic tissues. Pancreas was dissected and placed in 4% PFA for 24 hours at 4°C. Tissue was dehydrated in series of ethanols: 50%, 70%, 95%, 100%, and then in histoclear. Next, tissue was placed in histoclear and liquid hot paraffin wax 1:1, and allowed to cool and harden overnight at room temperature. The paraffin-infiltrated tissue was heated to 60°C. After melted, the tissue was incubated in fresh, hot paraffin for one hour. This was repeated 2 more times before embedding the tissue in paraffin in a block for sectioning. Tissue sections (10μm) were mounted on microscope slides. Standard immunohistochemistry procedures were followed: rehydration,
permeabilization with triton in PBS 0.04 %, block nonspecific binding with donkey serum 1:20, lastely incubate with primary antibodies overnight at 4°C, followed by secondary antibodies for 1 hour at room temperature. Tissues were stained for POMC and insulin or glucagon to identify if POMC expression in alpha or beta cells or in other cell types. The primary antibodies used were rabbit anti-ACTH (1:100), which can detect POMC and ACTH, mouse anti-glucagon, which can detect glucagon, mouse anti-insulin, which can detect insulin, and secondary antibodies were donkey anti-rabbit Alexa 555(1:800), and donkey anti-mouse Alexa 488(1:800).

In addition, immunofluorescence staining was performed on αTC1-6 cells and dispersed islets plated on chamber slides. Images were acquired and analyzed using Slide book software (Intelligent Imaging Innovation, Denver, CO) with a Zeiss Axiovert S 100 inverted microscope equipped with a z-stepper motor, Sutter filter wheels, Cooke Sensicam CCD camera, and a G4 Apple Macintosh computer.

Antigen retrieval

We optimized the staining procedure using antigen retrieval on pancreatic tissue. In order to detect some antigens, water bath treatment of paraffin sections is required, after the rehydration of paraffin sections, we rinsed them in distilled water before soaking
them in 200ml of 0.01M Citrate buffer (pH 6.0). Then, we put them in water bath at 90°C for 2×5 min; we refilled the jar with 40ml distilled water between the two treatments. After the second treatment, sections were left for 20-30 min in the buffer for cooling.

**Western Blot**

In order to detect POMC protein expression in alpha cells we used gel electrophoresis and Western blot techniques. We added 25-50μl of LDS (lithium dodecyl sulfate) to cell lysates and sonicate the lysate (apply ultrasound energy to disrupt cell membranes and release cellular contents). Next, we added 16μl of (LDS 235μl, DTT, dithiothreitol , 15μl) to cell lysates and boiled for 10 minutes. In NuPAGE 12% Bis-Tris Mini gels, ten wells were loaded with the following: αTC1-6 cell lysate, and control ATt-20 cell lysate, buffer, Magic marker and protein ladder. The gel system was subjected to electrophoresis in SDS running buffer at 25 mA for 10 minutes and then at 50 mA for 40 minutes. Proteins were transferred to nitrocellulose at 300 mA for 3 hours. We incubated the nitrocellulose paper with primary antibody anti- rabbit ACTH (1:1000) overnight at 4°C. The blot was then incubated with secondary antibody anti-rabbit Alexa floura 488 (1:1000) for 45 minutes at room temperature. Images were acquired using Molecular imager FX equipped with external laser, and a Sony computer.
**RNA isolation, cDNA Synthesis**

RNA was isolated using the protocol and reagents given in the Ambion RNAGueous-4PCR kit for isolation of DNA-free RNA. This protocol includes a treatment with DNase to remove any contaminating genomic DNA. RNA was then extracted and converted to first-strand complimentary DNA (cDNA) using the Invitrogen Superscript III First Strand Synthesis System for RT-PCR. Samples were prepared according to the First-Strand cDNA Synthesis protocol provided.

**Real- Time Quantitative (QPCR)**

Samples were prepared using the Applied Biosyste’s Pre-validated Taqman Gene Expression Assay Reagents and dye-labeled MGA primer probe. All were loaded on a 96-well plate and then amplified with the Q5 Multicolor Real-Time PCR Detection System iCycler, using IQ5 softwear. Expression levels were graphically analyzed using Kaleidagraph v4, and mRNA levels were reported as relative expression units compared with a cell-based standard. The standard used was a validated glyceraldehyde 3-phosphate dehydrogenase (GAPDH) standard. All material tested was from parallel reactions of reverse transcribed mRNA. GAPDH was verified to be an appropriate housekeeping gene in that it displayed constant expression relative to input the [RNA]
across all conditions test. The data were analyzed using unpaired Student’s t-test with equal or unequal variance as appropriate; t-values below 0.05 were considered to be statistically significant.
Results

POMC in alpha cells

Previous studies by Hochgeschwender et al. [2003] revealed alpha cells respond to $\alpha$-MSH through an unknown mechanism. In those studies POMC-null mice were given insulin to induce hypoglycemia but exhibited defective counterregulatory response, to return to euglycemia. When peripherally injected with $\alpha$MSH, the response was rescued. However, when these mice were injected with ACTH alone, the phenotype was not rescued. Thus, $\alpha$MSH plays an important role in glucagon secretion due to its ability to produce a counterregulatory response in alpha cells when injected during insulin tolerance tests (ITTs). A previous study showed that MC4R is expressed in islets as well as in $\alpha$-TC1-6 cells (Angleson, unpublished data). Therefore, $\alpha$-MSH acts through MC4R on alpha cells to trigger the glucagon secretion.

We determined the localization of POMC protein in mouse pancreatic tissue to establish which cells may be the source of the $\alpha$-MSH for alpha cell regulation. We utilized immunofluorescence on whole mouse pancreatic tissue stained with antibodies to
ACTH, glucagon, and insulin. Preliminary results revealed clear POMC staining in alpha cells and a weak signal in beta cells. We optimized the staining procedure using antigen retrieval on pancreatic tissue. Imaging showed a strong POMC signal in cells located in the periphery of islets but not in those cells in the interior [Figure 5]. Alpha cells are primarily located at the periphery of the islets, and beta cells at the core of the islets [Figure 4]. There was no POMC signal in negative control (data not shown).
Figure 4: Glucagon in alpha cells. Immunohistochemistry of mouse pancreatic tissue, showing the glucagon secreting (α) cells in green. DAPI stained cell nuclei in blue.
Figure 5: POMC protein is in alpha cells not in beta cells. Immunohistochemistry of mouse pancreatic tissue, showing anti-rabbit ACTH in red.
Because immunofluorescence of pancreatic tissue indicated POMC is expressed in alpha cells, we investigated whether POMC could be identified in αTC1-6 cells. The cell line exhibited positive staining for ACTH and glucagon [Figure 6]. There was no glucagon or POMC signal in negative control (data not shown).
Figure 6: POMC protein is expressed in αTC1-6 cells. Immunocytochemistry of αTC1-6 cells showing the glucagon secreting alpha cells in green, and the anti-rabbit ACTH in red.
To confirm the previous results, immunofluorescence was performed on dispersed pancreatic islets stained with anti-rabbit ACTH, anti-mouse glucagon, and anti-mouse insulin antibodies. Immunofluorescence showed clear αMSH staining in alpha cells (cells co-expressing glucagon), and lack of staining in beta cells (cells containing insulin) [Figure 7, 9]. There was no glucagon or POMC signal in negative control [Figure 8].
Figure 7: POMC protein in alpha cells. Immunocytochemistry of mouse dispersed islets showing glucagon secreting alpha cells in green and the anti-rabbit ACTH in red.
Figure 8: No POMC or glucagon signal. Immunocytochemistry of mouse dispersed islets showing glucagon secreting alpha cells in green and the anti-rabbit ACTH in red.
Figure 9: POMC protein lacking in beta cells. Immunocytochemistry of mouse dispersed islets showing insulin secreting beta cells in green and anti-rabbit ACTH in red.
To the results of POMC expression in alpha cells, a Western blot was used to detect the protein expression in alpha cells. αTC1-6 cell lysates and ATt-20 cells lysates (as a control) were used. The results showed a clear band around 20 kDa and 30 kDa both in αTC1-6 and the control. POMC molecular weight is 31 kDa and intermediate ACTH is 20 kDa. However, αMSH molecular weight is low (1.6 kDa) and would not be readily detected in this gel system [Figure 10].
Figure 10: Western blot shows POMC in aTC1-6. ATt-20 is a positive control.
The relative expression of POMC in islets versus αTC1-6

The results of the present study indicate that POMC is expressed in αTC1-6 cells and primary alpha cells. A previous study utilizing PCR analysis detected POMC gene both in islet and αTC 1-6 samples (Angleson, unpublished data). Therefore, quantitative PCR (Q PCR) was used to measure the gene expression of POMC in αTC 1-6 and mouse islets. Results showed POMC level was below the detection in αTC 1-6 cells; however, a low level of POMC expression was detected in islets [Figure 11].
Figure 11: POMC expression in islets vs αTC1-6. Pituitary and AtT-20 are a positive control.
**Discussion**

This study determined that POMC is expressed in pancreatic alpha cells but not in beta cells. Thus alpha cells are an autocrine source of α-MSH. Previous studies performed by the Angleson Lab provided more support. First, the melanocortin receptor (MC4R) is located in primary alpha cells and is therefore likely responsible for the counterregulatory response exhibited during ITTs. Second, melanocortin receptors (MC4R) stimulate glucagon secretion through the cAMP pathway. Third, the melanocortin α-MSH was found to increase glucagon gene transcription and expression. Fourth, there is an increase in intracellular calcium [Ca2+]i by α-MSH in α-TC1-6 cells.

We can conclude from this study that POMC is expressed by pancreatic alpha cells. Although QPCR showed that POMC was below the detection level in αTC1-6, POMC-derived end products are not the primary protein secreted by alpha cells. Previous QPCR analysis done by the Angleson Lab compared the glucagon expression level between islet and αTC1-6. They found that glucagon was 29X higher in islets than in
αTC1-6 cells. However, keeping in mind that alpha cells make up approximately 15-20% of islets, this result indicates that glucagon is roughly 145X-193X higher in primary alpha cells than in α-TC1-6 cells.

This finding indicates that POMC acts as an autocrine signal. POMC binds to autocrine receptors (MC4R) on alpha cell leading to glucagon secretion. However, we do not currently have information about POMC processing in alpha cells. Western blot showed two bands, one at 20 kDa and the other at 30 kDa representing ACTH and POMC, respectively. We know that αMSH not ACTH has rescued the phenotype after ITTs. Further analysis is necessary to determine the identity of POMC end products in alpha cells. However, an early study on mouse pancreatic islet cell types has shown that alpha cells synthesize PC2 to mediate the posttranslational processing of proglucagon, but do not synthesize PC1/3 (Marcinkiewicz et al. 1994). In another study, S. tropicalis POMC cDNA (wild-type) was transiently transfected into the alpha cell line α-TC1.9 (different from α-TC1-6). Two days after transfection, the steady-state levels of α-MSH-related and β-endorphin-related end-products in transfect α-TC1.9 cells were nearly the same as the steady-state levels of these POMC-related end-products in
lysates of the S. tropicalis intermediate pituitary (Dores et al. 2011). Thus previous study provided insight into the end products of POMC processing in alpha cells. We would predict that POMC processing in alpha cells will produce α-MSH and β-endorphin.

We propose a model for intrinsic regulation of glucagon secretion. Acute hypoglycemia leads to an increase in intracellular calcium producing weak glucagon and POMC secretion. POMC and glucagon activate the MCR4 and glucagon receptors, respectively (autocrine receptors in alpha cells) leading to strong glucagon secretion. In contrast, during chronic hypoglycemia (starvation) free fatty and amino acids increase in the blood. This leads to the inhibition of paracrine factors, as well as strong stimulation of glucagon secretion.

The results of the current study suggest another mechanism for regulating glucose homeostasis, and another approach for treating diabetes mellitus and related glycemic dysregulation. As POMC plays an important role in controlling glucagon secretion, researchers can investigate possible disturbances in POMC expression in alpha cells and relations to uncontrolled blood sugar levels. For example, if alpha cells overexpress POMC, this can lead to increased glucagon secretion and hyperglycemia. Therefore, we need to further elucidate POMC processing and the role of POMC in alpha cells function in human pancreatic tissue.
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