Published in final edited form as:
Mol Cell Endocrinol. 2010 August 30; 325(1-2): 36–45. doi:10.1016/j.mce.2010.05.008.

Characterization of the Expression, Localization, and Secretion of PANDER in α–Cells

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Abstract

The novel islet-specific protein PANcreatic DERived Factor (PANDER; FAM3B) has beenextensively characterized with respect to the β–cell, and these studies suggest a potential function for PANDER in the regulation of glucose homeostasis. Little is known regarding PANDER in pancreatic α–cells, which are critically involved in maintaining euglycemia. Here we present the first report elucidating the expression and regulation of PANDER within the α–cell. *Pander* mRNA and protein aredetected in α–cells, with primary localization to a glucagon-negative granular cytosolic compartment. PANDER secretion from α–cells is nutritionally and hormonally regulated by L-arginine and insulin, demonstrating similarities and differences with glucagon. Signaling via the insulin receptor (IR) through the PI3K and Akt/PKB node is required for insulin-stimulated PANDER release. The separate localization of PANDER and glucagon is consistent with their differential regulation, and the effect of insulin suggests a paracrine/endocrine effect on PANDER release. This provides further insight into the potential glucose-regulatory role of PANDER.

Keywords

Alpha Cells; α–TC1-6; FAM3B; Glucagon; Insulin; Islet; PANDER; Secretion

1. Introduction

PANcreatic DERived factor (PANDER, FAM3B) is a 235 amino acid protein which is one of four members of a novel family of cytokine-like proteins identified by a computational genomic search as previously described (Zhu *et al*., 2002). PANDER shares approximately 31.6 – 53.3% primary sequence homology with the other members of the FAM3 family

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The material contained in this manuscript was used for the partial fulfillment of the Ph.D. dissertation requirement for J.R.C. at the University of Pennsylvania in the laboratory of Dr. Bryan Wolf.

(FAM3A, C and D), but without extensive homology to any other known cytokines. PANDER is predominantly expressed in the pancreatic islets of Langerhans, with minimal expression in the small intestine and prostate (Zhu *et al*., 2002).

PANDER is robustly expressed in the β–cells of islets and various β–cell lines (Cao *et al*., 2003;Burkhardt *et al*., 2005;Yang *et al*., 2005). Localization and regulatory similarities exist between PANDER and insulin in β–cells, including moderate co-localization within insulin granules (Xu *et al*., 2005) and co-secretion in response to glucose challenge (Burkhardt *et al*., 2005). The *pander* promoter is positively regulated by glucose in β–cell lines and islets (Burkhardt *et al*., 2005), with dose-dependent induction of *pander* mRNA and protein expression (Wang *et al*., 2008;Yang *et al*., 2005). Our limited characterization of a recently created global PANDER knockout mouse model reveals aberrations in homeostatic mechanisms including impaired glucose tolerance, insulin secretion, and reduced hepatic glucose production (HGP) (Burkhardt *et al*., 2009). Both *in-vitro* and *in-vivo* data generated thus far suggests a potential role for PANDER in glucose homeostasis.

Much of the work by our group and others has focused on PANDER expression and regulation in β–cell lines and islets with primary focus on similarities to insulin (Burkhardt *et al*., 2005;Burkhardt *et al*., 2008;Yang *et al*., 2005;Xu *et al*., 2005;Wang *et al*., 2008). However, PANDER is also detected in both α–cell lines and primary α–cells, possibly at greater levels in α– than in β–cells (Burkhardt *et al*., 2005;Cao *et al*., 2003). As the second largest islet cell population, α–cells are critically involved in the maintenance of euglycemia, primarily via secretion of the peptide hormone glucagon (FOA *et al*., 1952;FOA *et al*., 1957). Glucagon is released mainly in response to hypoglycemia in the fasted state *in vivo*, but is also regulated in response to other nutritional, hormonal, and neuroendrocrine stimuli (Gromada *et al*., 2007). Acting chiefly at the liver, glucagon antagonizes the action of insulin, with hyperglucagonemia and elevated hepatic glucose production observed in the diabetic state (Baron *et al*., 1987;Reaven *et al*., 1987). This underscores the physiological relevance of α–cells, glucagon, and potentially PANDER in maintaining normoglycemia.

With little known regarding PANDER in α–cells, we present the first report demonstrating robust PANDER expression within the pancreatic α–cell, differential cytoplasmic localization with respect to glucagon, and regulation in response to L-arginine, insulin, and glucose challenge, with mechanistic insight into insulin-stimulated PANDER release.

2. Materials and Methods

2.1 Cell Culture

α–TC1-6 (courtesy of Dr. Bruce Verchere, University of British Colombia, Canada), α–TC3, α–TC-Lenti-IR (courtest of Dr. Michael Wheeler, University of Toronto, Canada), and C2C12 (ATCC) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, 12491-015) while βTC3 cells were maintained in RPMI-1640 medium (Invitrogen, 12633-012), both supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37°C, 95% air, 5% CO_2. Cells were passaged weekly and were used at passages 26-45.

2.2 Antibodies

Detection of endogenous and over-expressed PANDER by immunoblot and immunohistochemical labeling of PANDER in pancreatic sections was performed using rabbit polyclonal PANDER anti-serum (Alpha Diagnostics, San Antonio, TX). For immuno-fluorescent (IF) labeling, the following antibodies were used: anti-glucagon monoclonal (R&D Systems, MAB1249); anti-glucagon polyclonal (Millipore, 4031-01); anti-FLAG (M2) polyclonal (Cell Signaling Technology, 2368); anti-GM130 monoclonal (BD

Transduction Labs, 612008); anti-PDI (Affinity Bioreagents, MA3-019); anti-LAMP-1 (Developmental Studies Hybridoma Bank, DSHB 1D4B), and anti-Chromogranin-A polyclonal (Novus Biologicals, NB110-2475). Secondary antibodies were either Alexa-Fluor dye conjugated [DAR-Alexa 488; DAM-Alexa 594; DAGP-Alexa 647], biotinylated [DAR-biotin, DAM-biotin], or Cy-conjugated [DAM-Cy3, DAR-Cy2]. Tertiary streptavidin-HRP or streptavidin-Alexa-dye conjugates were employed for sequential LSAB-IHC / IF. For assessment of insulin signaling, the following antibodies were employed: pAkt-Thr308 (Cell Signaling, 4056), pAkt-Ser473 (Cell Signaling, 4060), and Akt (Cell Signaling, 9272).

2.3 Transient transfections and nucleofections

Adherent α–TC1-6 cells were transiently transfected with 4 μg PANDER plasmid using Lipofectamine 2000 (Invitrogen, 11668-019) in OPTI-MEM (Invitrogen, 31985-062) according to manufacturer instructions. α–TC1-6 cells in suspension were nucleofected with 2 μg pCMV-PANDER-3-FLAG using the nucleofector kit V (Amaxa Biosystems, VCA-1003) and program G-016 according to manufacturer instructions.

2.4 Cell lysate preparation and western blotting

Adherent cell layers were rinsed with cold Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen, 14040-117) and lysed using Roth's Lysis Buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mg/ml aprotinin, and 0.01 mg/ml leupeptin), or 1X Cell Lysis Buffer (Cell Signaling, 9803) supplemented with phosphatase inhibitor cocktail 2 (Sigma, P5726) and 1 mM PMSF. Lysates were stored at −20°C until SDS-PAGE analysis.

For immunoblots, equal quantities of total lysate protein were boiled for 5 minutes in 5X SDS-sample buffer with 15 mg/ml dithiothreitol (DTT) and resolved on 12% polyacrylamide gels. Protein transfer to nitrocellulose or PVDF membranes was carried out and blotted using antibodies listed above and detection performed using ECL Advance Western blotting kit (GE Healthcare, RPN-2135). Immunoblot visualization was perfomed using the FujiFilm LAS-3000 Imaging system. Densitometric analyses were performed using the FujiFilm Multigauge software package.

2.5 Murine islet isolation, dispersion and FACS-sorting

Murine pancreatic islets were isolated from 6-10 week old C57BL/6 mice as previously described (Gao *et al*., 1999). Islets were hand-picked, counted, and dispersed using 1.8 U/ml dispase in Hank's Balanced Salt Solution (HBSS) and gentle trituration. The resulting single-cell suspension was then sorted by fluorescence-activated cell sorting (FACS) as outlined previously (Pipeleers *et al*., 1985a).

2.6 RNA isolation from cell lines and primary islet cell populations

Adherent cell layers were rinsed in cold DPBS, lysed, and RNA isolated using the RNeasy kit (QIAGEN, 74104) according to manufacturer instructions. DNAse treatment of all samples was performed. Equivalent input RNA was used for quantitative Taqman® RT-PCR. For *insulin*, *glucagon*, and *β–actin* amplification, commercially available Gene Expression Assays were used (Applied Biosystems), while for *pander*, either commercially available or the primer /probes listed below were employed. PANDER forward – 5′-TGCTCGCGGAGCTCATTC 3′, PANDER reverse – 5′-CCAATGCTTCGGATGTTGTAGA-3′, fluorescent (FAM) PANDER probe – 5′-TGACGTGCCCCTGTCCAGCACT-3′.

Primary α, β and non–α/non–β cell enriched populations obtained by FACS sorting of murine islets (as outlined above) were lysed and RNA isolated using the Taqman® PreAmp

Cells-to-C_T kit (Ambion, 4387299). 500 ng of input RNA was reverse transcribed and pre-amplified for target transcripts. Pre-amplification products were subsequently used in Taqman® qPCR.

2.7 Immuohistochemical labeling of murine islets

Pancreata isolated from male C57BL/6 mice were fixed overnight in 1:10 buffered formalin (Fisher Scientific, SF93-4). Tissue was then processed, paraffin embedded, and 4 μm sections mounted onto ProbeOn™ Plus slides (FisherBiotech, 15-188-52) and slides stored at room temperature until stained. The sections were labeled for PANDER and glucagon using sequential LSAB-DAB and LSAB Immuno-fluorescent staining respectively. Slides were visualized using a confocal microscope (Leica, DM IRE2) coupled to a spectral confocal system (Leica, TCS SP2) and micrographs captured.

2.8 Generation of PANDER-3-FLAG construct

The coding sequence of the *pander* gene was cloned upstream (5′) of the triple-FLAG repeat moiety of the pCMV-3FLAG-3a plasmid (Stratagene, 240197), employing *EcoR*V and *Xho*I restriction endonuclease sites within the multiple cloning region and construct integrity was confirmed by restriction digest and sequence analysis.

2.9 Immuno-fluorescent labeling of α–TC1-6 cells

α–TC1-6 cells nucleofected with pCMV-PANDER-3FLAG (described above) were cultured on glass coverslips (Fisherbrand®, 12-545-82-12CIR-1D) in complete DMEM. 48 hrs post-nucleofection, cells were fixed in 4% paraformaldehyde, then blocked using PSG: a solution of 0.01% saponin, 0.25% fish skin gelatin and 0.02% NaN_3 in 1X PBS. Cells were then incubated in primary antibodies specific for FLAG and various sub-cellular markers using antibodies diluted in PSG for 1 hr at room temperature in a humidified environment. Coverslips were then rinsed three times with PSG at room temperature. Secondary antibodies in PSG were added to coverslips for 1 hr and cells again rinsed with PSG. Cells were then counterstained with DAPI and coverslips mounted using MOWIOL and stored at 4°C in the dark until visualized, and confocal micrographs captured.

2.10 PANDER secretion from α–cell lines

α–TC1-6 and α–TC6-Lenti-IR cells were transiently transfected with pShuttle-PANDER (as previously outlined). 24 hrs post-transfection, cells were rinsed and pre-incubated in DMEM, 3 mM glucose, 1% FBS for 1 hr prior to stimulation with L-Arginine (Sigma, A5006), Insulin (Sigma, I9278), or a combination of both at the indicated concentrations. For insulin stimulation experiments, cells were serum starved at 1% FBS for at least 5 hrs prior to stimulation. Where indicated, 200 nM wortmannin (Sigma, W1628) in DMSO was added to pre-incubation media prior to insulin stimulation. Post-stimulation, media was collected and cells lysed. Harvested media was concentrated by ammonium sulfate protein precipitation (0.55 g/ml media). Equal quantities of media or lysate protein were resolved by SDS-PAGE and probed for PANDER. Densitometric analysis was carried out as previously described and relative secreted PANDER determined as ratio of PANDER in media to that in lysate for each sample, and expressed as fold over negative control.

2.11 Hormone secretion

Glucagon secreted into culture media from α–TC1-6 cells was determined using Glucagon EIA (Alpco, 48-GLUHU-E01) or RadioImmunoAssay (Linco, GL-32K) kits according to manufacturer instructions and normalized to total protein content of cell lysates.

2.12 Data and statistical analyses

Statistical analyses were performed using the GraphPad Prism 5.0 software package. Statistical significance of differences was determined by Student's *t*-test or analysis of variance (ANOVA) where more than two groups were compared. Threshold of statistical significance defined as $P<0.05$.

3. Results

3.1 Qualitative evaluation of PANDER in α–TC1-6 and primary α–cells in-situ

To investigate the expression of PANDER in pancreatic alpha cells, the α–TC1 subclone 6 (α–TC1-6) glucagonoma cell line was chosen as a suitable model as it secretes glucagon exclusively, compared with other α–cell lines (such as α–TC3) which also secrete low levels of insulin (Hohmeier & Newgard, 2004). Qualitative determination of PANDER expression in these cells using immunofluorescent labeling revealed a diffuse, often peripheral profile with some similarity to the granular, mostly peripheral pattern of glucagon staining observed here typical of α–cell lines (**Fig. 1**A – C). While some overlap of PANDER and glucagon is observed by wide-field microscopy (**Fig. 1**C, inset), specific sub-cellular localization of PANDER within the α–TC1-6 cell could not be determined using this approach. Nonetheless, immunofluorescent labeling demonstrates that PANDER is robustly expressed in this α–cell line. As expected, similarly labeled PANDER-null C2C12 murine myoblast cells stained negatively for both PANDER and glucagon (**Fig. 1**D – F).

To assess PANDER expression in intact α–cells *in-situ*, we performed sequential immunohistochemical / immunofluorescent labeling of paraffin-embedded murine pancreatic sections for PANDER and glucagon respectively. This approach was employed to overcome limitations of antibody sensitivity for endogenous PANDER detection by immunofluorescence in sectioned tissue. We observed robust PANDER labeling (yellow) within both the glucagon-negative, centrally located β–cells, and the peripheral population of glucagon-positive (magenta) α–cells indicated by overlap of PANDER and glucagon signals (**Fig. 1**G – I).

3.2 Quantitative evaluation of PANDER in α–cell lines and primary islet cell populations

With qualitative evidence of PANDER expression in both α–cell lines and primary islets, quantitative assessment was performed, using a TaqMan® RT-PCR approach to measure *pander* mRNA in α–TC1-6, β–TC3, and the C2C12 cell lines. Similar levels of *pander* transcript were detected in the α–TC1-6 and β–TC3 cell lines, with no expression observed in C2C12 cells (**Fig. 2**A). Additionally, at the protein level, PANDER expression was evaluated by densitometric analysis of western immunoblots of lysates harvested from the α–TC1-6 and β–TC3 cells. When normalized to β–actin, we observed similar levels of PANDER in these islet cell lines (**Fig. 2**B). We note however, that there is a non-statistically significant trend to lower PANDER protein expression in the β–TC3 cell line as compared with α–TC1-6 cells, despite highly similar mRNA content.

Islets comprise roughly 1% of total pancreatic area, with β–cells representing 70 – 75%, α–cells 15 – 20%, and δ and ε–cells comprising the remaining <10% of islet mass. The generation of enriched populations of primary murine islet α–, β–, and non–α, non–β cells by different sorting techniques is therefore challenging, with maximum cellular yields of about 10% of starting material. The low number of α–cells obtained by islet sorting therefore precluded reliable assessment of PANDER protein expression in these primary cells. However, using the technique described by Pipeleers in 1985 (Pipeleers *et al*., 1985a), *pander* mRNA in islet cell fractions enriched in α–, β–, and non–α, non–β cells was measured. Using this approach we observe that *pander* is more highly expressed in the α–

cell enriched population compared with both the β–cell and non–α, non–β cell enriched fractions. This approximate 6-fold difference is statistically significant ($P < 0.05$) (**Fig. 2**C). We also observed significantly higher PANDER expression in the non–α non–β cell enriched fraction compared to the β–cell population, most likely due to contamination of this fraction with residual α–cells from the sorting procedure. TaqMan® RT-PCR detection of *insulin* and *glucagon* transcripts in the three populations indicates the expected enrichment with *glucagon* and *insulin* expression predominantly confined to the α–cell, and β–cell enriched fractions respectively ($P<0.0001$ for both) (**Fig. 2**C). Overall, the data suggest that PANDER is similarly expressed in β– and α–cell lines, but more robustly expressed at the mRNA level in primary α–cells as compared with β–cells of murine islets.

3.3 Sub-cellular localization of PANDER within α–TC1-6 cells

The sub-cellular localization of secretory proteins, including hormones, provides insight into both regulatory mechanisms and biological function. As PANDER regulation is largely uncharacterized in α–cells, localization within α–TC1-6 cells specifically with respect to glucagon was investigated. Currently available PANDER antibodies lack the sensitivity required for discrete sub-cellular analysis. In order to circumvent this problem and facilitate precise PANDER detection, a C-terminal PANDER-3-FLAG fusion protein construct was generated (**Fig. 3**A).

Expression of the fusion protein in lysates of transiently transfected α–TC1-6 cells 24 hrs post-transfection was demonstrated by immunoblot (**Fig. 3**B). Both cleaved and un-cleaved forms of the fusion protein are detected in the lysates of transfected cells using both FLAG and PANDER polyclonal antibodies with absence of signal in the lysates of non-transfected cells (**Fig. 3**B). Secretion of the fusion protein into culture media of transfected cells was separately confirmed (data not shown).

To evaluate discrete PANDER localization, simultaneous co-labeling of PANDER-FLAG nucleofected α–TC1-6 cells for FLAG, glucagon, and various markers for sub-cellular compartments was performed (**Fig. 3**C – N). Based on nucleofection efficiency; only about 40% of cultured cells expressed the construct at sufficient levels to observe robust FLAG labeling. It is not surprising therefore that within a typical cell cluster not all cells label positively for FLAG expression. The markers used include Chromogranin-A, for neuroendocrine vesicle identification, GM-130 to label the cis-Golgi network, and protein disulfide isomerase (PDI), which labels the lumen of the endoplasmic reticulum (ER). Confocal micrographs demonstrate that while both PANDER-FLAG and glucagon localize to granular cytoplasmic compartments, minimal overlap of the two is observed (**Fig. 3**C – E). Modest overlap between FLAG and chromogranin-A is observed in these cells (**Fig. 3**F – H), however co-localization is observed between PANDER-FLAG and GM130 (cis-Golgi) (**Fig. 3**I – K), and PDI (endoplasmic reticulum) (**Fig. 3**L – N). Together, these data suggest that PANDER localizes predominantly to a glucagon-negative granular cytoplasmic compartment downstream of its processing in the Golgi and ER.

3.4 L-Arginine-stimulated PANDER secretion from α–TC1-6 cells

To further characterize PANDER in α–cells, the regulation of PANDER secretion in response to glucose, amino acids, and insulin was investigated. These nutrient and hormonal cues have been demonstrated to represent important α–cell secretagogues and inhibitors respectively (Gromada *et al*., 2007). Electrogenic entry of positively charged amino acids (such as arginine) through cationic transporters induces membrane depolarization, thus triggering glucagon release (Gromada *et al*., 2007). Due to the technical limitations of detecting and reliably measuring secreted PANDER, it was necessary to examine PANDER release using time-points determined by previous studies at which significant accumulation

in culture media is expected (Yang *et al*., 2005). When stimulated with a dose-curve of arginine over 16 hrs, we observe robust stimulation of PANDER secretion from transfected α–TC1-6 cells at 5 mM, becoming and remaining statistically significant between 10 and 20 mM arginine (**Fig. 4**A). While there appears to be an influence of arginine on PANDER content, the slight variability observed in cell lysate PANDER expression more directly reflects the transfection efficiency, as expression is not driven by the endogenous PANDER promoter. The doublet observed on immunoblot for PANDER reflects alternate cleavage sites within the signal peptide of full-length PANDER. As such, both bands of the doublet are considered in densitometric analysis. As a control for cell function, secreted glucagon was also measured (Fig. 4B). We observed significant glucagon release in response to 15 and 20 mM arginine, with non-significant stimulation at lower concentrations. In attempting to characterize arginine-stimulated PANDER secretion over time, we observed consistent basal secretion beginning at 2 through 24 hrs of continuous stimulation and identified a significant effect of arginine on PANDER release by two-way ANOVA (Arginine, $P<0.004$; Time, $P<0.0002$), (**Fig. 4**C). It is difficult to comment on the significance of this observation as we lack suitable methods of detecting secreted PANDER at early time-points. Earlier detection of PANDER, perhaps within 15 – 20 minutes post-stimulation would be ideal, but is not currently feasible using currently available antibodies. This may however reflect rapid release of PANDER in response to arginine challenge, consistent with the proposed mechanism of arginine induced exocytosis as via membrane depolarization. Despite minimal co-localization of PANDER and glucagon, both are positively regulated by arginine, indirectly indicating localization of PANDER to a secretory compartment.

3.5 Regulation of PANDER secretion from α–TC1-6 cells by glucose

One of the most physiologically relevant stimuli influencing α–cells is glucose, with hypoglycemia inducing significant glucagon release (Gromada *et al*., 2007). Whether this effect is entirely direct, or partially indirect via de-repression as β–cell paracrine effector levels decline *in-vivo* remains elusive. However, α–TC1-6 cells have been shown to secrete glucagon in response to acute hypoglycemia (Hohmeier & Newgard, 2004). When exposed to increasing or decreasing concentrations of glucose, basal PANDER secretion from transfected α–TC1-6 cells remains unchanged, without any stimulatory or inhibitory effect on PANDER observed (data not shown).

3.6 Regulation and potential mechanism of PANDER secretion from α–TC1-6 cells by insulin

Interesting roles are currently debated for β–cell secretory products in the regulation of α–cell secretion. Chief among these factors is the glucagon antagonist insulin. Insulin is proposed to have a direct inhibitory effect on glucagon secretion from α–cells, particularly in the context of elevated local glucose concentration (Bailey *et al*., 2007;Xu *et al*., 2006). In addressing a potential inhibitory effect of insulin, on arginine-stimulated PANDER release, we exposed PANDER-transfected α–TC1-6 cells to 17 nM insulin, 20 mM L-arginine or a combination of both. Insulin significantly stimulated PANDER release, with apparent synergy observed in combination with arginine (**Fig. 5**A). In these studies there was a concordant increase in both PANDER and glucagon secretion and in response to 20 mM L-arginine. Interestingly, insulin had a robust stimulatory effect on PANDER release, and we observed an expected inhibition of glucagon secretion. In combination, insulin effectively suppressed arginine-induced glucagon release, while stimulated PANDER secretion remained.

With the unexpected positive regulation of PANDER by low levels of insulin, we focused our investigations on elucidating the mechanism by which insulin achieves this effect. Insulin exerts its metabolic roles by signaling through the insulin receptor (IR) complex via

phosphoinositide-3-kinase (PI3K) and the Akt/PKB node (Taniguchi *et al*., 2006). We therefore assessed the requirement for signaling through PI3K and Akt for insulin-regulated PANDER secretion. We employed both biochemical and genetic approaches. Initially, we assessed PANDER secretion in the presence of the specific PI3K inhibitor wortmannin. When pre-treated with wortmannin, insulin-stimulated Akt phosphorylation at Ser 473, was significantly attenuated compared with control lysates, as expected (**Fig. 5**B). Insulin-regulated PANDER release was significantly reduced in stimulated cells pre-treated with wortmannin as compared with control cells (**Fig. 5**C). These results demonstrate that signaling through PI3K is essential for insulin-regulated PANDER release.

To further assess the requirement for signaling through the insulin receptor complex via the Akt node for insulin-stimulated PANDER secretion, we employed α–TC6 cells stably expressing a lentivirally-encoded anti-sense hairpin targeted to the β–chain of the insulin receptor. These cells were generated and characterized by the Wheeler group at the University of British Colombia (Diao *et al*., 2005). These cells have reduced insulin receptor expression (Diao *et al*., 2005) which we confirmed (data not shown), and in response to insulin-challenge demonstrate reduced Akt activation as compared with control cells (Fig. 6A) (Diao *et al*., 2005). On exposure to a similar dose-curve of insulin as used for wortmannin-treated cells, insulin-induced PANDER secretion was significantly attenuated in α–TC-IR cells, particularly at 17 nM (Fig. 6B). At 17 nM the insulin-stimulated increase no longer achieved statistical significance, while at 50 nM, secretion is diminished as compared with control α–TC1-6 cells (**Fig. 6**B). Therefore, using both genetic and biochemical approaches, we have demonstrated that signaling through IR–β via PI3K and Akt is necessary for insulin-regulated PANDER secretion from α–cells.

Overall, our report characterizing the expression, distribution, and regulation of PANDER in pancreatic α–cells indicates robust expression and localization of this novel protein to a granular compartment discrete from glucagon, which is positively regulated in response to insulin stimulation.

4. Discussion

Despite considerable progress in the characterization of PANDER, the biological function remains unknown. Current evidence indicates a putative role in regulating glucose homeostasis. Indeed, the transcriptional activity and secretion of PANDER protein from the pancreatic β–cell are dose-dependently regulated by glucose (Burkhardt *et al*., 2005;Yang *et al*., 2005;Wang *et al*., 2008). The predominant restriction of PANDER to the glucagon- and insulin-positive cells of the pancreatic islet, the chief organ of gluco-regulation, provides indication of its potential role (Zhu *et al*., 2002). Furthermore, both PANDER mouse models (PANDER transgenic – TG, and knockout – PANDER $^{-/-}$) reveal multiple gluco-regulatory aberrations (Burkhardt *et al*., 2009;Robert *et al*., 2006).

The islet-specific PANDER over-expressing transgenic mouse model (TG) displays high-fat-induced glucose intolerance, elevated hepatic glucose production, and impaired hepatic insulin signaling. Targeted disruption of PANDER in a global knockout mouse model results in impairment of glucose tolerance, stimulated insulin release, and diminished hepatic glucose production (Burkhardt *et al*., 2009). Recent evidence also demonstrates that PANDER binds to liver membrane and attenuates proximal steps of the insulin signaling cascade in human liver-derived HepG2 cells (Yang *et al*., 2009). Taken together, the various studies suggest that PANDER may serve as a novel islet-derived hormone which acts distally at the liver to modulate insulin action. However, to further understand how PANDER exerts its peripheral effects, it is necessary to provide additional biological characterization with regard to its expression and regulated secretion. Our present study

provides further clues addressing regulated PANDER release from the previously neglected α–cell population.

Secretion from pancreatic α–cells is robustly stimulated by decreasing glucose concentrations (hypoglycemia) *in-vivo*, amino acid challenge, islet paracrine effectors, and other neuroendrocrine stimuli (Gromada *et al*., 2007). In contrast, elevated glucose and insulin concentrations are proposed to individually and synergistically suppress glucagon release, with potential roles attributed to novel β–cell factors such as γ–aminobutyric acid (GABA). Our studies demonstrate distinct localization of PANDER and glucagon within α–cells, which is concordant with the observed differential regulation of the two. Increasing and decreasing glucose concentrations exerts no influence on PANDER release, while there is a robust stimulatory effect of L-arginine. Further contrasting with glucagon regulation is insulin-induced PANDER secretion from α–cells, which is dependent on signaling through the insulin receptor complex downstream of PI3K and Akt/PKB. The distinct localization of PANDER and glucagon, coupled with insulin-induced PANDER release and targeting to the liver, is consistent with a role for PANDER in the modulation of hepatic insulin activity and glucose homeostasis (Yang *et al*., 2009).

Greater *pander* mRNA expression within α–cells as compared to β–cells suggests that the biological role of PANDER may be more relevant to these cells. All neuroendocrine cells including pancreatic α– and β–cells contain at least two types of secretory vesicles: the dense-core glucagon and insulin-containing granules respectively, and smaller synaptic-like microvesicles (SLMV's) (Moriyama *et al*., 1996;Thomas-Reetz & De, 1994;Regnier-Vigouroux & Huttner, 1993). In β–cells, SLMV's contain GABA, while in α–cells they appear to contain glutamate as suggested by the presence of membrane-bound glutamate transporters (Gaskins *et al*., 1995;Llona, 1995;Wiedenmann *et al*., 1988). The regulated secretion of both GABA and glutamate is emerging as a potential modulatory mechanism for islet paracrine function with peripheral effects on glucose homeostasis (Xu *et al*., 2006;Cabrera *et al*., 2008;Bertrand *et al*., 1992;Bertrand *et al*., 1993;Moriyama *et al*., 2000;Hayashi *et al*., 2003;Llona, 1995). While the regulation of SLMV's has been more thoroughly investigated in β–cells than in α-cells, the exact nature and contents of α–cell SLMV's are largely unknown. It has been shown in β–cells that despite significant responses to the same secretagogues there is differential regulation of GABA-containing SLMV's compared to the dense-core insulin granules. The mechanisms by which these microvesicles are regulated are not well understood, but appear to involve both constitutive and regulated secretory pathways. The regulatory similarities and critical differences observed with PANDER and glucagon are concordant with their discrete localization within the pancreatic α–cell, and both basal and stimulated PANDER release. Taken together, this implies the potential localization of PANDER to SLMV's within these cells.

The current lack of sensitive in-house and commercially available PANDER antibodies has hindered our ability to adequately detect endogenous PANDER protein. This has warranted the use of both transient transfection for over-expression and epitope (FLAG)-tagging of PANDER for stringent detection by western analysis and confocal immunofluorescent microscopy, respectively. This approach for characterizing both protein expression and localization has been extensively utilized by a number of groups without demonstrating protein mis-localization due to over-expression or presence of the (FLAG) epitope (Bhattacharyya *et al*., 1995;Olkkonen *et al*., 1993;Lipman *et al*., 1998;Duan *et al*., 1995;Advani *et al*., 1998). By employing this approach we are therefore confident that the expression of the fusion protein and its discrete localization within a granular compartment separate from glucagon in α–TC1-6 cell has been successfully demonstrated.

Pancreatic islets are highly vascularized and innervated, allowing their constituent cells to respond cooperatively to achieve and maintain normoglycemia in the balancing of energy demand and nutrient availability. While the factors influencing glucagon are not well understood, a number of nutritional, hormonal, and neural stimuli have been implicated (Gerich *et al*., 1976;Pipeleers *et al*., 1985b). There is also considerable evidence supporting the paracrine/endocrine theory of α–cell function, underscoring the influence of β–cell secretory products on glucagon release (Gromada *et al*., 2007). Given the disparate localization of glucagon and PANDER, it is not surprising that the two are differentially regulated by insulin. While signaling through PI3K and Akt has been shown to be inhibitory to glucagon release (Diao *et al*., 2005), this signal cascade robustly induces PANDER secretion. Once released into the circulation, PANDER likely targets distal sites such as the liver to exert its normal biological function (Yang *et al*., 2009). As a subtle modulator of hepatic insulin action, PANDER released from α–cells may act *in lieu* of or cooperatively with glucagon to enhance hepatic glucose production.

Progression to overt type 2 diabetes occurs via increasing peripheral insulin resistance, and overt disease is marked by hyperinsulinemia resulting from β–cell compensation, and hyperglucagonemia due to α–cell dysfunction, concomitant with the hyperglycemia resulting from decreased insulin sensitivity, increased glucagon action and endogenous glucose production. The relevance of α–cell function and hepatic glucose production to chronic hyperglycemia is progressively emerging as a critical component in our understanding of the underlying pathologies. What is highly intriguing yet requires further investigation is that various parameters of metabolic syndrome such as hyperglycemia, and hyper-insulinemia have now been shown to induce PANDER secretion from pancreatic β– and α–cells, respectively. Coupled with a potential role in modulating insulin action, it is reasonable to speculate that PANDER may possibly serve as a co-factor in the onset and subsequent progression of metabolic syndrome by exacerbating hepatic insulin resistance.

In summary, we have demonstrated the robust expression of PANDER within pancreatic α–cells and its localization to a discrete vesicular cytoplasmic compartment apart from glucagon. We have also elucidated both nutritional and hormonal regulation of PANDER release from α–cells. We observe no effect of glucose on PANDER secretion, while both L-arginine and insulin are robustly stimulatory. Further, insulin-stimulated PANDER secretion is dependent on signaling through the insulin receptor, downstream of PI3K and via the Akt node. These and other data from previous studies together suggest that PANDER released into systemic circulation may exert distal effects at the liver to modulate insulin action and hepatic glucose production, thereby influencing glycemia.

Acknowledgments

The authors would to acknowledge Dr. Edward Williamson and the Pathology Core of the Children's Hospital of Philadelphia for invaluable assistance with confocal imaging studies. This publication was made possible through core services provided by the DERC at the University of Pennsylvania (DK 19525). This work was supported by grant K01-DK070744 (to B.R.B.) from the NIDDK, National Institutes of Health, and the Juvenile Diabetes Research Foundation (to B.A.W.).

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases (DK070744 to B.R.B.) and the Juvenile Diabetes Research Foundation (JDRF).

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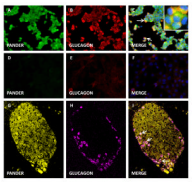

Fig. 1.

Qualitative evaluation of PANDER in α–TC1-6 and primary α–cells *in-situ*. (A) PANDER (green), and (B) glucagon (red)-specific immuno-fluorescent labeling of α–TC1-6 cells with merge of PANDER, glucagon, and DAPI-stained (blue) nuclei indicated in yellow (C). High-magnification inset illustrates observed overlap of PANDER and glucagon signals in these cells. PANDER (green) (D), glucagon (red)-specific labeling (E) of C2C12 cells, with merge of signals (F). All cells were cultured for 48 hrs under basal conditions prior to fixation using 4% paraformaldehyde / DPBS. (G-I) Sequential immuno-histochemical and immuno-fluorescent labeling of a murine pancreatic section for PANDER and glucagon respectively. Confocal, DAB (PANDER, yellow) labeling (G) was captured under bright-field illumination, and fluorescent glucagon (magenta) signal (H) was captured under excitation at 594 nm. Merge of PANDER and glucagon signals shown in (I). Arrows indicate representative regions of observed overlap indicated in pink. Images are representative of at least three independent experiments.

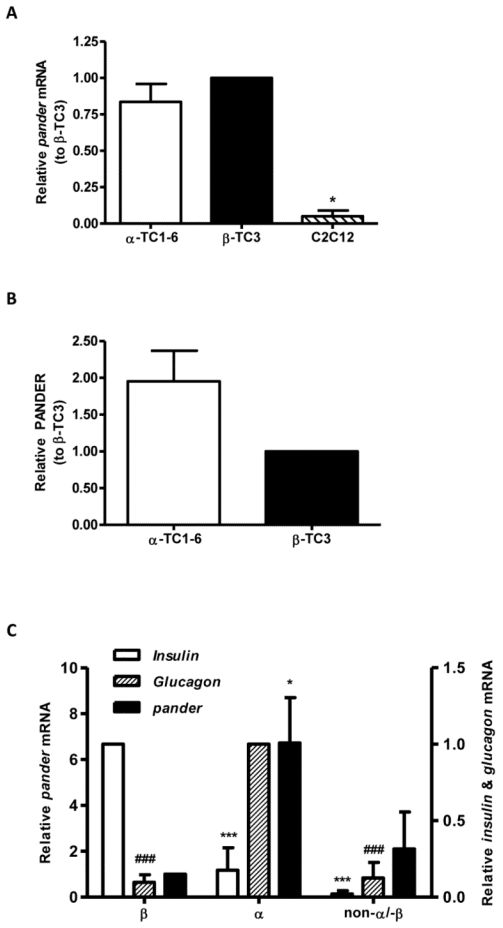

Fig. 2.

Quantitative evaluation of PANDER in α–cell lines and sorted islet cell populations. (A) Relative levels of *pander* mRNA were determined by Taqman® qPCR in total RNA isolated from the α–TC1-6, β–TC3, and C2C12 cell lines cultured under basal conditions. (B) Relative levels of PANDER protein in cell lysates (normalized to β–actin) of α–TC1-6 and β–TC3 cell lines as determined by densitometric analysis of denaturing PANDER western blot. In each case, levels of PANDER mRNA and protein are expressed as fold relative to that in β–TC3 cells. (C) Murine islets were dispersed into single-cell fractions and FACS sorted to yield enriched populations of α–, β–, and non–α / non–β cells as described. Relative levels of *pander* (C; left y-axis), *glucagon* (C; right y-axis), and *insulin* (C; right y-axis) transcript were assessed by Taqman® qPCR using the $2^{-\Delta\Delta CT}$ method. *, $P<0.05$, ***, $P < 0.001$ compared with β–cells, and ###, $P < 0.001$ compared with α–cells. All data are presented as means ± SEM of at least three independent experiments.

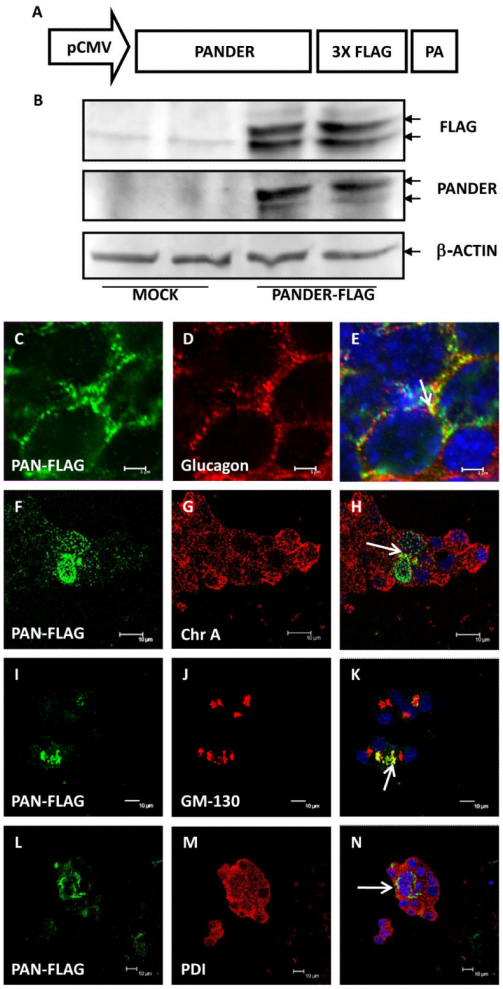

Fig. 3.

Sub-cellular localization of PANDER within α–TC1-6 cells. (A) The PANDER coding sequence was cloned into the pCMV-3FLAG (Stratagene) expression vector immediately upstream of the triple FLAG repeat sequence to generate a C-terminal fusion-protein construct as shown. (B) FLAG *(upper)* and PANDER-specific *(middle)* immunoblots of lysates from transiently transfected α–TC1-6 cells harvested 48 hrs post-transfection are shown. As a loading control, β–actin content was assessed (*lower panel*). (C – N) Confocal immunofluorescent micrographs of PANDER-FLAG nucleofected α–TC1-6 cells 48 hrs post-nucleofection co-labeled for FLAG and various sub-cellular markers: FLAG-specific labeling shown in (C, F, I, and L). Glucagon-specific (D), Chromogranin-A specific (G), Golgin-97-specific (J), and protein disulfide isomerase (PDI)-specific (M) co-labeling of nucleofected cells, with merge of signals shown (in E, H, K, and N). Images presented are representative of at least three independent experiments. Arrows indicate representative regions of co-localization (yellow).

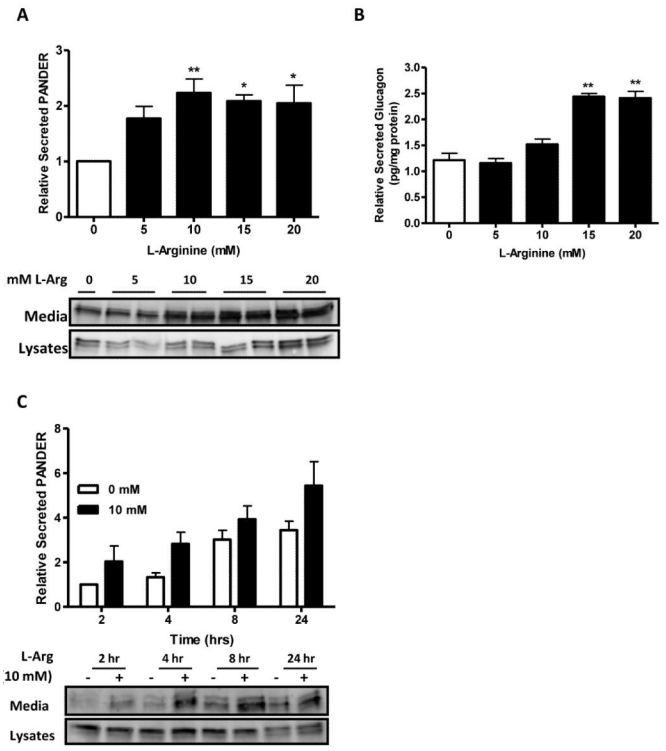

Fig. 4.

L-Arginine-stimulated PANDER secretion from α–TC1-6 cells. (A) Transiently transfected α–TC1-6 cells were pre-incubated at 3 mM glucose and stimulated with increasing concentrations of L-arginine for 16 hrs. Relative secreted PANDER, normalized to cellular PANDER content, was determined by densitometric analysis of denaturing immunoblots and expressed as fold over mock-stimulated control (*upper panel*). Representative media and cell lysate blots for PANDER are shown below (*lower panel*). (B) Relative glucagon secreted into culture media following dose-escalating arginine stimulation was measured and normalized to cellular protein content and expressed as fold compared with mock-stimulated control. (C) Time-course evaluation of arginine-stimulated PANDER secretion between 2 and 24 hrs, with representative PANDER media and lysate blots shown below. All data shown are representative of at least five independent experiments. *, $P < 0.05$; **, $P < 0.01$.

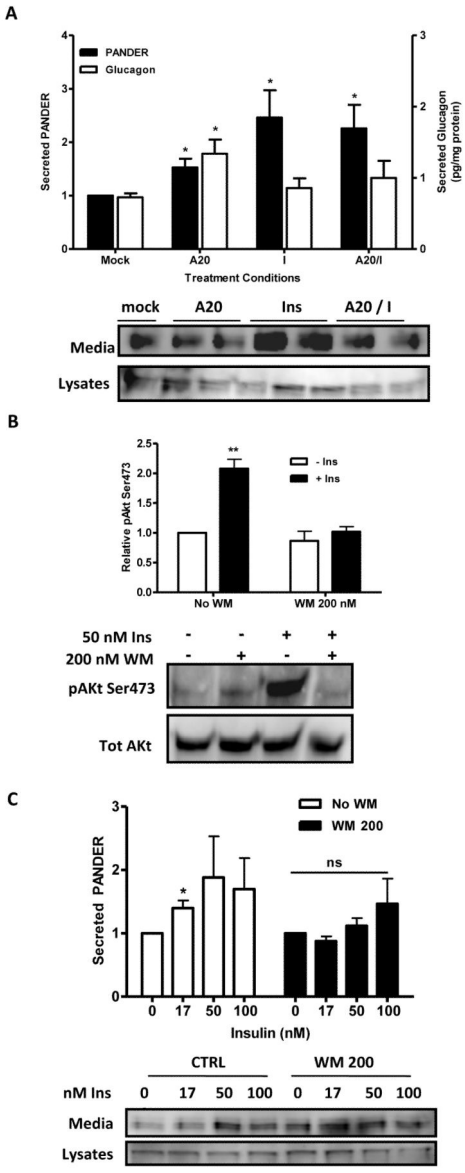

Fig. 5.

L-Arginine and Insulin-stimulated PANDER secretion from α–TC1-6. (A) Assessment of PANDER and glucagon secreted from transiently PANDER-transfected α–TC1-6 cells in response to 16 hr challenge with 20 mM L-Arginine, 17 nM insulin, or a combination of both. PANDER secreted into culture media, normalized to cellular PANDER content of lysates, was determined by densitometric analysis of immunoblots and presented as fold compared with mock-stimulated control. All stimulations were performed in duplicate dishes. Representative media and lysate blots shown below. Glucagon secreted into culture media over the period of arginine or insulin stimulation was measured by radioimmunoassay. (B) Insulin-stimulated Akt activation (Ser 473 phosphorylation) in α–TC1-6 cells pre-treated with 200 nM wortmannin and stimulated with 50 nM insulin was assessed by western immunoblot. Representative blots shown below. (C) Dose-escalation insulin-stimulated PANDER secretion from α–TC1-6 cells with and without wortmannin pre-treatment. Secretion was measured 16 hrs post-stimulation and expressed as fold relative

to un-stimulated control. Representative media and lysates blots shown below. All data presented is representative of at least five independent experiments. * $P < 0.05$ according to either Student's *t*-test or ANOVA relative to control mock condition in each case.

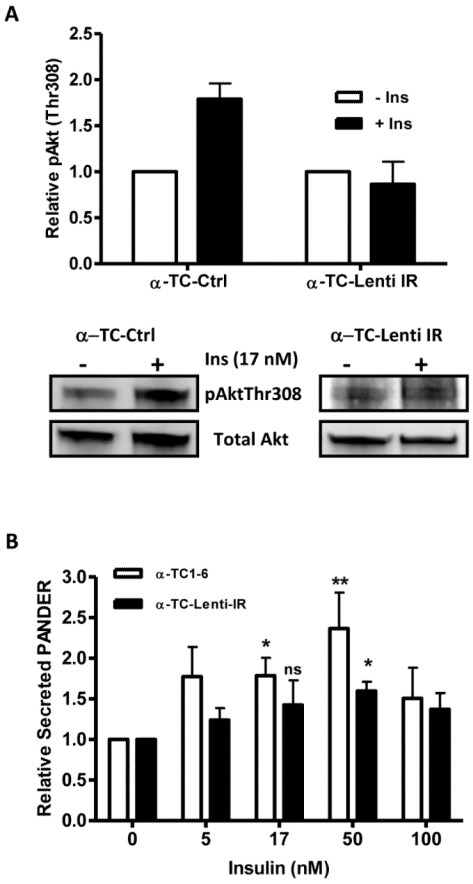

Fig. 6.

Insulin-stimulated PANDER secretion from α–TC-Lenti-IR cells. (A) α–TC-Lenti-IR and control cells were serum starved at 1% FBS for 5 hrs prior to stimulation with 50 nM insulin for 15 mins. Akt activation (phosphorylation at the Thr308 residue) was determined by immunoblot and normalized to total Akt. Representative blots are shown below. (B) Transiently transfected and serum starved α–TC-Lenti-IR cells were stimulated with increasing insulin concentrations for 16 hrs and PANDER in culture media and cell lysates assessed by immunoblot, and densitometric analysis, and compared with secretion from control α–TC1-6 cells.* $P < 0.05$; ** $P < 0.01$ relative to control mock condition in each case. All data presented are representative of at least three independent experiments.