Apelin is a novel islet peptide.

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Abstract

Apelin, a recently discovered peptide with wide tissue distribution, regulates feeding behavior, improves glucose utilization, and inhibits insulin secretion. We examined whether apelin is expressed in human islets, as well as in normal and type 2 diabetic (T2D) animal islets. Further, we studied islet apelin regulation and the effect of apelin on insulin secretion. Apelin expression and regulation was examined in human and animal specimens using immunocytochemistry, in situ hybridization, and real-time PCR. Insulin secretion was studied in INS-1 (832/13) clonal beta cells. APJ receptor expression was studied using real-time PCR. In human and murine islets apelin was predominantly expressed in beta cells and alpha cells; a subpopulation of the PP-cells in human islets also harbored apelin. In porcine and feline islets apelin was mainly expressed in beta cells. APJ-receptor expression was detected in INS-1 (832/13) cells, and in human and mouse islets. A high dose (1μM) of apelin-36 caused a moderate increase in glucose-stimulated insulin secretion (30%; $p<0.001$), while lower concentrations (10-100nM) of apelin robustly reduced insulin secretion by 50% ($p<0.001$). Apelin was upregulated in beta cells of T2D db/db mice (47 % vs controls; $p<0.02$) and GK-rats (74% vs controls; $p<0.002$), but human islet apelin expression was unaffected by glucose. On the other hand, human islet apelin expression was diminished after culture in glucocorticoids (16% vs. controls; $p<0.01$). We conclude that apelin is a novel insulin-regulating islet peptide in humans and several laboratory animals. Islet apelin expression is negatively regulated by glucocorticoids, and upregulated in T2D animals. The presence of apelin receptors in islets suggests a role for apelin as a paracrine or autocrine messenger within the islets.

INTRODUCTION

Apelin is a recently identified peptide hormone that turned out to be the endogenous ligand of the previously orphan receptor APJ [1]. APJ was identified by O´Dowd et al [2] and remained orphan until Tatemoto et al, in 1998 identified apelin [1]. The peptide was given the name apelin for, APJ receptor ligand [1, 3]. Several molecular forms, e.g. apelin-13, apelin-17, and apelin-36, are cleaved from the 77-amino-acid preproapelin precursor [1, 3-5].

In the central nervous system of both humans and rats, mRNA encoding APJ receptor and apelin are widely distributed, suggesting a role for apelin in central regulatory pathways [6-10]. In peripheral tissues, APJ and apelin expression has been shown in e.g. adipose tissue, lungs, heart and mammary glands [3, 6, 8-12]. Apelin has also been localized to endothelial cells of small arteries in several organs [13]. Apelin is involved in a broad range of physiological functions, e.g. fluid homeostasis [9, 14], regulation of food intake [14, 15], and angiogenesis [16]. Furthermore, apelin has been shown to have hypotensive properties, lowering both systolic and diastolic blood pressure [9, 13, 17] and increase heart rate [7, 18]. A potent positive inotropic action of apelin has been demonstrated in rat hearts in vitro [19] and in vivo [20]. Moreover, apelin and APJ may have a role in pathophysiology of human heart failure [17, 21] and plasma concentrations of apelin are decreased in patients suffering from parenchymal lung disease with preserved cardiac function [22].

Since apelin was originally isolated from bovine stomach extracts [2], a function in the gastrointestinal tract was expected. In rat stomach, apelin-positive cells were identified by immunohistochemistry as mucous neck- parietal- and chief cells [23]. Further apelin was shown to be expressed in human and rodent colon [24], and apelin was shown to stimulate gastric cell proliferation in vitro, and to stimulate CCK secretion from a murine

enteroendocrine cell line [25]. Apelin is produced in adipocytes in humans and mice [26], indicating that apelin could be an adipokine. Furthermore, insulin stimulates adipocyte apelin production both in vivo and in vitro, and apelin expression in adipocytes was inhibited by fasting and recovered after refeeding [26]. Interestingly, Sörhede Winzell et al [27], demonstrated that apelin-36 decreased glucose-stimulated insulin secretion (GSIS) in mice, both in vivo and in vitro. Moreover, expression of APJ receptor mRNA in isolated mouse islets was demonstrated [27]. A positive correlation between plasma insulin and apelin in both humans and mice has been shown [26, 28]. Further, basal and 2 h post glucose plasma levels of apelin are elevated in type 2 diabetic (T2D) subjects and in and humans with impaired glucose tolerance [29]. Furthermore, plasma apelin is increased in obese and hyperinsulinemic mice [26]. On the other hand, Erdem et al [30] and Zhang et al [31] found circulating apelin levels to be lower in T2D patients. Interestingly, recent studies by Dray et al [32] showed that apelin lowers plasma glucose via increased glucose utilization in fat and muscle. Taken together, a role for apelin as a regulator of insulin or glucose levels seems likely.

Many regulatory peptides are expressed within the islets of Langerhans, in islet cells or nerve fibers innervating the islets, and regulate islet hormone secretion via paracrine mechanisms. Among such peptides are e.g. IAPP [33], NPY [34], PYY [35], ghrelin [36], and CART [37]. Based on this knowledge, we studied the possibility of apelin expression in the pancreatic islets of humans and laboratory animals, using immunocytochemistry and in situ hybridisation. In addition, to study a possible impact of T2D on islet apelin expression, we included rodent models of T2D. Furthermore, the effect of apelin on insulin secretion was examined using clonal beta cells. Our data show high expression of apelin in human islets and in islets of all laboratory animals examined. Apelin had a dual, concentration-dependent effect on insulin secretion and human islet apelin expression is regulated by glucocorticoids.

MATERIALS AND METHODS

Tissue and tissue processing

Human pancreatic specimens (adult: n=10, fetal, 18-22 weeks of gestation: n=5) were taken during autopsy or during pancreatic surgery. The studies were approved by the Human Ethics Committee in Lund. The specimens were immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 at 4°C, rinsed in graded ethanols, and embedded in paraffin. Sections (6μm thickness) were mounted on slides, deparaffinized, and hydrated before further handling. Male GK-rats (n=5, age 20w), Wistar rats (n=5, age 20w), db/db mice (n=5, age 16w), and wild type (WT) C57Bl/6J mice (age 16w n=5, postnatal day (P) 3: n=8) were supplied by Taconic A/S Ry, Denmark. Pigs (n=5) and cats (n=5) were killed for other purposes. All animals were fed ad libitum at sacrifice. Further, GK-rats and Wistar control rats, as well as db/db mice and C57Bl/6J control mice were killed at the same time of the day to ensure the same nutritional status in T2D model animals as in control animals. Murine, porcine, and feline specimens were dissected out, fixed overnight in Stefanini's solution (2% paraformaldehyde and 0.2% picric acid in 0.1M phosphate buffered saline, pH 7.2), rinsed thoroughly in Tyrode's solution containing 10% sucrose, and frozen on dry ice. Sections (10μm thickness) were cut and thaw-mounted on slides. The project was approved by the Animal Ethical Committee in Lund and Malmö.

Immunocytochemistry

Antibodies were diluted in phosphate buffered saline (PBS) (pH 7.2) containing 0.25% bovine serum albumin and 0.25% Triton X-100. Paraffin sections were boiled in a microwave oven in a 0.01M citrate buffer (pH 6.0) for 2x7 min at 650 W. Sections were incubated with primary antibodies overnight at 4° C, followed by rinsing in PBS with Triton X-100 for 2 x 10

min. The following primary antibodies were used: rabbit polyclonal anti-apelin, raised against the evolutionary conserved C-terminal decapeptide of human apelin-36 (Friis-Hansen, Gøtze and Rehfeld, Dept of Clin. Biochemistry, University of Copenhagen, Copenhagen, Denmark), dilution 1:400; rabbit polyclonal anti-apelin, raised against the human/rodent shared 13-amino-acid region of the C-terminus of apelin, dilution 1:400 [26]; goat polyclonal anti-apelin, dilution 1:50, code SC33469 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), guinea pig polyclonal anti-proinsulin, dilution 1:5120, code 9003 (EuroDiagnostica, Malmö, Sweden) [38]; guinea pig polyclonal anti-glucagon, dilution 1:5120, code 8708 (EuroDiagnostica) [36], rabbit polyclonal anti-somatostatin, dilution 1:800, code N-SOM, (DiaSorin Inc., Stillwater, MN, USA) [39]; sheep polyclonal anti-pancreatic polypeptide, dilution 1:640, code AHP 515 (Serotec, Oxford, UK) [36]; goat polyclonal anti-ghrelin, dilution 1:1000, code SC10368 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) [40]. Thereafter secondary antibodies with specificity for rabbit-, guinea pig-, goat-, or sheep- IgG, and coupled to either fluorescein isothiocyanate (FITC), AMCA, or Texas-Red (Jackson, West Grove, PA, USA), were applied on the sections. Incubation was for 1h at room temperature. Sections were again rinsed in Triton X-100 enriched PBS for 2 x 10 min and then mounted in PBS:glycerol, 1:1. The specificity of immunostaining was tested using primary antisera pre-absorbed with excess amount of homologus antigen (100μg of peptide per ml antiserum in working dilution), or by omission of primary antibodies.

In situ hybridization

A synthetic oligodeoxyribonucleotide probe, complementary to the sequence 2291-2320 of human apelin mRNA (Accession number NM_017413), was used. The probe was 3 '-endtailed with [^{35}S]dATP (Perkin Elmer, Stockholm, Sweden). The in situ hybridization protocol has been described previously [33]. In brief, deparaffinized and hydrated sections

were treated with proteinase K (10 μg/ml, Sigma, St. Louis, MO, USA) for 30 min at 37°C, fixed in 4% paraformaldehyde for 15 min, washed for 5 min in PBS, and then acetylated with 0.25% acetic anhydride in 0.1M triethanolamine for 10 min. Thereafter sections were dehydrated in graded ethanols, and air dried. Hybridization was carried out in sealed moisturizing chambers at 37°C overnight, using a probe concentration of approximately 1 pmol/ml, followed by stringent post-hybridization washing (1X SSC; 0.15 M NaCl, 0.015 M sodium citrate). The slides were dipped in Ilford K.5 emulsion and stored in light sealed boxes at 4°C for 21 days. They were then developed in Kodak D-19, fixed in Kodak polymax and mounted in Kaiser's glycerol gelatine. Controls sections were hybridized in the presence of 100-fold excess of unlabelled probe.

Imaging and morphometry

Immunofluorescence was examined in an epi-fluorescence microscope (Olympus BX60). In situ hybridization was analyzed in bright-field. Images were taken with a digital camera (Nikon DS-2Mv). For analysis of the ratio of beta cells expressing apelin, images of all islets (n=5-15) within three sections from different parts of pancreas were taken for each animal, as previously described [41]. Areas of immunostaining in digitized images were analyzed using Biopix iQ software (BioPixAB, Göteborg, Sweden). First, the insulin stained area for each islet was delineated and calculated, thereafter the apelin stained area within the insulin stained area was calculated. The identity of the specimens was unknown to the observer both when images were taken, and when data were analyzed.

Culture of human islets and real time PCR for apelin and APJ mRNA.

Human islets were obtained from the Nordic Network for Clinical Islet Transplantation and isolated as previously described [42]. The islets were cultured for 48 hours in 3.3 mM

glucose, 16.7 mM glucose, or 3.3 mM glucose with the addition of 6.25 nM dexamethasone. RNA was isolated with the AllPrep DNA/RNA mini kit (80204, Qiagen, Hilding, Germany), and reverse-transcribed to cDNA with the first strand cDNA synthesis kit (K1612, Fermentas, Helsingborg, Sweden). Real-time PCR was performed for apelin (Hs00936329_m1, Applied Biosystems, Foster City, CA), human APJ receptor (Hs00766613_m1), rat APJ receptor (Rn00580252_s1) and mouse APJ receptor (Mm00442191_s1 (Applied Biosystems) and human cyclophilin A (4310883E,), rat HPRT (Rn01527838_g1) and mouse HPRT (Mm03024075_m1) were used as endogenous controls. The results were analyzed by the ΔΔCt method.

Cell culture

Rat insulinoma INS-1 (832/13) cells [43] at population doublings 59-82, were cultured in RPMI-1640 medium, containing 11.1 mM glucose and supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM β-mercaptoethanol, and 100 U/ml penicillin and 100 μg/ml streptomycin at 37ºC in a humidified atmosphere, containing 95% air and 5% CO_2.

Insulin secretion assay

Cells were seeded in 24-well plates and grown to confluence. The cells were washed and pre-incubated for two hours in HEPES-balanced salt solution (HBSS) containing (mM) 114 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.16 $MgSO_4$, 20 HEPES, 25.5 $NaHCO_3$, 2.5 $CaCl_2$ at pH 7.2 and with 0.575 BSA and 2.8 glucose at 37ºC. The buffer was replaced by HBSS containing glucose and the indicated concentrations of apelin-36 or apelin-13 (cat.no 057-28, and 057-29 respectively, Phoenix Pharmaceuticals, Birlingame, CA) for 1 hour at 37ºC. The buffer was carefully removed and the amount of insulin was determined with mouse insulin ELISA kit

(cat.no 10-1149, Mercodia, Uppsala, Sweden), which cross-reacts with rat insulin to 146% and human insulin to 195%. The cells were washed with PBS and stored at -20ºC until determination of protein content, which was measured with the bicinchoninic acid method (Pierce Chemical Co, Rockford, IL).

Statistics

All data were analyzed using student's un-paired t-test or a one-way ANOVA followed by Bonferroni's post hoc test. Data are presented as mean±SEM. Differences with a p-value<0.05 were considered significant.

RESULTS

Apelin in human islets.

Immunocytochemistry for apelin revealed that apelin immunoreactive (IR) cells were abundant in pancreatic islet cells in both fetal (Fig 1A and Fig 2 D-F) and adult humans (Fig 2). Importantly, the same staining pattern was seen with three different antibodies against apelin. Preabsorption of the apelin antisera with excess of apelin-36 peptide blocked all staining in both islets (Fig 1) and pituitary, which was used as a positive control (data not shown). To identify the apelin IR islet cells we performed double and triple immunostainings for apelin and the main islet hormones (insulin, glucagon, somatostatin, ghrelin, and PP). In all human specimens studied, apelin was co-expressed with insulin in beta cells (Fig 2A-C) and with glucagon in alpha cells (Fig 2D-F). Quantification revealed that 95±5% of both alpha cells and beta cells harbored apelin. Apelin was not found in the somatostatin-producing delta cells (Fig 2G-I). The majority of the PP cells were devoid of apelin, but a few cells harboured both peptides (Fig 2J-L). Ghrelin cells were consistently devoid of apelin (Fig 2M-O). There were no differences in cellular identity of the apelin IR cells between fetal and adult

human specimens. To confirm the immunocytochemistry data, in situ hybridization for apelin mRNA was performed on human pancreatic sections. Also apelin mRNA was abundantly expressed in human islets in a pattern similar to that of apelin IR, i.e labelling was seen in both central and peripheral parts of the islets (Fig 3A). Absorption controls displayed scattered background labelling only (Fig 3B). Labelling for apelin mRNA was also seen in human mammary glands and pituitary, that were used as positive controls (data not shown). To further verify apelin expression in human islets we performed real-time PCR for apelin mRNA on isolated human islets (n=4). Also these experiments showed that apelin was highly expressed in human islets (Fig 4). The expression level was similar to that of cyclophilin A, which was used as an endogenous control. In adipoctyes, apelin expression has been shown to be unaffected by glucose [26]. To investigate if islet apelin mRNA is regulated by glucose or not, human islets were cultured for 48h in 3.3 or 16.7 mM glucose respectively. There was no effect of glucose on islet apelin mRNA expression (Fig 4A). Furthermore, since apelin has been shown to be regulated by glucocorticoids in adipocytes [26, 44], we cultured islets in 6.25 nM dexamethasone and 3.3 mM glucose for 48 h. Addition of dexamethasone robustly decreased apelin mRNA to 16$\pm$2% (Fig 4B) of that of the control islets cultured in parallel ($p<0.01$).

Apelin in mouse, rat, cat, and pig islets.

To corroborate the finding of apelin in human islets, and to search for suitable animal models to study islet apelin regulation, we next examined whether apelin is expressed also in the islets of four different laboratory animal species. Apelin IR was seen in numerous islet cells in both adult and neonatal (P3) mice. Double immunostainings for apelin/glucagon, apelin/insulin and apelin/somatostatin revealed expression of apelin in both alpha cells and beta cells (Fig 5), but not in delta cells. This was evident in both adult and neonatal mice.

Similarly, apelin was also found in both alpha and beta cells also in rats (Fig 5). The number of apelin IR islet cells in rodents was generally lower than in human islets. In porcine (Fig 6A-C) and feline islets (Fig 6D-F), apelin was predominantly expressed in beta cells. A few apelin IR cells were devoid of insulin IR; double staining for glucagon revealed that these cells were alpha cells (data not shown).

Apelin affects insulin secretion from clonal beta cells.

Apelin-36 has been shown to inhibit GSIS from mouse islets in vitro and in vivo in mice [27]. Since isolated mouse islets consist of at least 4 different cell types (beta cells, alpha cells, delta cells, and PP-cells) conclusions about direct effects on the beta cells cannot be made. Hence, the effect of apelin directly on the beta cell has hitherto not been studied. Therefore, we examined the effect of apelin-36 on GSIS in INS-1 (832/13) clonal beta cells. Apelin-36 was chosen since this form of apelin was shown to affect insulin secretion in mice [27]. Raising the glucose concentration in the culture medium from 2.8 mM glucose to 16.7 mM glucose provoked a 7.3±0.8 fold increase in insulin secreted to the medium during the 1 h static incubation (2.8 mM glucose: 45.0±4.7 vs. 16.7 mM glucose: 299.2±9.9 ng/mg protein/hr, $p<0.001$). At high glucose, addition of 10 nM and 100 nM apelin-36 caused a decrease in insulin secretion by approximately 50% to 136.9±11.2 and 140.2±20.5 ng insulin/mg protein/hr ($p<0.001$), whereas addition of 1nM apelin-36 had no effect on insulin secretion (287.2±31). When adding a higher dose (1 μM) of apelin-36, GSIS was instead moderately augmented by approximately 30% to 378.0±25.5 ng insulin/mg protein/hr ($p<0.05$). At low glucose, addition of apelin-36 had no effect on insulin secretion. The results are summarized in Fig 7. When apelin-13 was used in the same concentrations and under the same conditions, we were unable to detect any effects on insulin secretion (data not shown).

APJ is expressed in human and mouse islets and INS-1 (832/13) cells.

Since we found apelin-36 to affect insulin secretion from INS-1 (832/13) cells we wanted to investigate whether these cells also express the APJ receptor. To this end real time PCR was employed. These experiments revealed that INS-1 (832/13) cells express APJ at a moderate level. Next we performed real-time PCR for APJ on isolated mouse and human islets. These analyses confirmed previously reported data on APJ expression in mouse islets [27] and revealed that APJ is expressed also in human islets.

Apelin is upregulated in islets of rodent T2D-models.

Our data showing that apelin affects insulin secretion, prompted investigation of a possible impact of T2D on islet apelin expression. Since apelin was found to be expressed in both rat and mouse islets we studied rodent models of T2D; db/db mice and GK-rats. As in normal control mice and rats, apelin was found in both alpha cells and beta cells in both db/db mice and GK-rats. Further, apelin was not seen in delta cells or PP-cells. Quantification of apelin IR beta cells, revealed that both T2D models had moderately higher levels of apelin than the control animals. Thus, db/db mice displayed 47% more beta cells with apelin IR than WT mice (db/db 50$\pm$5% vs. WT 34$\pm$4%; p=0.017), and GK-rats had 74% more apelin positive beta cells than Wistar control rats (GK-rats 47$\pm$4% vs. Wistar rats 27$\pm$4%, p=0.0019). The results are summarized in Figure 8.

DISCUSSION

The islets of Langerhans are key regulators of metabolism and failure of islet function plays a central role during development of T2D. The islets harbour and are regulated by a plethora of regulatory peptides. Increased knowledge about these peptides, where they are expressed and

what actions they exert, in normal physiology and in T2D, will increase the understanding about islet hormone regulation and hopefully lead to new treatment strategies for T2D. Apelin is a recently identified hormone with wide tissue distribution. Interestingly, apelin has been shown to inhibit insulin secretion [27] and to improve glucose homeostasis [32]. Hitherto, apelin expression in the islets has not been demonstrated. Further, the effect of apelin directly on beta cells has not been studied.

Here we show that apelin-36 exerts a dual effect on insulin secretion from clonal beta cells, and that apelin peptide and mRNA is expressed in human islets, as well as in islets of four laboratory animal species. Thus, apelin was found in beta cells and alpha cells in human, mouse and rat islets. In porcine and feline islets, apelin was mainly expressed in beta cells. Further, APJ expression was found in human and mouse islets, as well as in clonal beta cells. Furthermore, we show that apelin is upregulated in the beta cells of two mechanistically different rodent models of T2D, and that human islet apelin mRNA is diminished by glucocorticoids.

Our present data on clonal beta cells show that apelin regulates insulin secretion via a direct action on the beta cell. Effects of apelin on insulin secretion were only evident at high glucose concentrations, suggesting that the effect is glucose-dependent. Further, the effect of apelin on insulin secretion was complex. A high dose (1μM; towards pharmacological) apelin caused an augmentation of GSIS. However, at lower (10-100nM) and more physiological doses, i.e. doses likely to be achieved within the islets, apelin inhibited GSIS. It should be mentioned that lower doses than this (1nM) had no effect on GSIS. Our data showing that the inhibitory effect was glucose dependent is in agreement with Sörhede Winzell et al [27] who were unable to detect any effect of apelin on 2.8 or 8.3 mM glucose, but detected an inhibitory

effect of apelin at 16.7 mM glucose. The APJ receptor is coupled to Gi-proteins and exerts its effects by decreasing cAMP [5]. We find APJ receptor expression in INS-1 (832/13) clonal beta cells as well as in human and mouse islets. This pathway could thus explain our data on the inhibitory effect of apelin. We have, however, no mechanistical explanation for the stimulatory effect. Whether receptors other than APJ mediate the stimulatory effect of apelin needs further investigation. It should be mentioned that Sörhede Winzell et al reported that a dose of 1μM apelin-36 inhibited insulin secretion from isolated mouse islets [27]. In the present report we show inhibitory effects of apelin at lower doses (10nM and 100nM). The difference could be species-related or perhaps be explained by disturbance of APJ receptors expression in islets caused by the isolation procedure, or by differences in the expression levels of the receptor. Moreover, we show in the present report that 1μM apelin-36 stimulates GSIS from clonal beta cells. There is no ready explanation for the divergent data. Whether apelin also regulates islet hormones other than insulin is not known, and further studies are warranted to elucidate this.

Although we find that apelin is upregulated in hyperglycemic db/db mice and GK-rats, and others have shown that plasma apelin is increased after an oral glucose load [29], our present data does not favor any major role for glucose as a regulator of apelin expression in human islets, since culturing human isolated islets in different concentrations of glucose had no effect on apelin expression. Our data in islets are reminiscent of previous data in adipocytes, in which apelin expression was not affected by glucose [26]. The present finding that apelin mRNA is decreased by glucocorticoids in isolated human islets is also in line with previous data in adipocytes [44], in which apelin expression is also negatively regulated by glucocorticoids.

We found apelin and APJ-receptor expression in a similar fashion in the islets of several different mammalian species. This suggests that apelin is an evolutionary conserved islet signaling molecule. The production of apelin in islet cells together with our present and previously reported data on APJ receptor expression in islets [27] suggest the existence of a APJ/apelin regulatory system within the islets. Thus, apelin secreted from islet cells could act as a paracrine and/or autocrine regulator of insulin secretion. Even though we find a dual effect of apelin on insulin secretion we believe that it is the effect seen at physiological doses, i.e. inhibition of insulin, that is the predominating effect in the islet. Importantly, this interpretation is supported by the reported inhibitory action on insulin secretion in mice [27]. Interestingly, Wei et al [44] and Boucher et al [26] showed that adipocyte apelin expression is positively regulated by insulin. Provided the same action of insulin on apelin expression in islet cells, it could be hypothesized that islet apelin acts as a negative feedback signal to inhibit insulin secretion in situations of high insulin levels. Our data on increased apelin expression in islets of hyperinsulinemic T2D animal models (db/db mice and GK-rats) points in this direction. This is reminiscent of the insulinostatic peptide NPY which is robustly overexpressed in rats made T2D after treatment with high doses of glucocorticoids [34]. Our data on upregulation of apelin in models of T2D gain support from a study showing that plasma levels of apelin are elevated in type T2D subjects and in and humans with impaired glucose tolerance [29]. Furthermore, plasma apelin is increased in obese and hyperinsulinemic mice [26]. Admittedly, other reports [30, 31] showed that circulating apelin levels are lower in T2D patients. Since apelin improves glucose utilization in skeletal muscle and adipose tissue [32], one could hypothesize that beta cell apelin is upregulated in order to improve glycemia in the target tissues via hormonal routes.

In summary, this study shows that apelin is a novel regulator of insulin secretion expressed in human, mouse, rat, pig and cat islets. Islet apelin is regulated by glucocorticoids, but not by glucose. Further, apelin is upregulated in animal models of T2D, possibly to inhibit excess secretion of insulin. The APJ receptor is expressed in islets and a role for apelin as a paracrine or autocrine messenger within the islets seems likely.

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FIGURE LEGENDS

Figure 1. Immunofluorescence photomicrographs of sections of human fetal pancreas. **A:** Staining for apelin in islets. **B:** The same islets as in A in a consecutive section. Negative control staining using apelin antibodies preabsorbed with apelin-36.

Figure 2. Immunofluorescence photomicrographs of sections of human pancreas from adults (A-C, G-O) and fetus (D-F). Double immunostaining for apelin and insulin (A-C), apelin and glucagon (D-F), apelin and somatostatin (G-I), apelin and PP (J-L), apelin and ghrelin (M-O). Arrows exemplify coexpression. Note that apelin is present in the majority of all beta cells

and alpha cells, but not in delta cells or ghrelin cells. A subpopulation of the PP cells harbour apelin. Scale bar=50µm.

Figure 3. In situ hybridization autoradiographs of human pancreatic islets. **A:**. Islet with labelling for apelin mRNA. Arrows exemplify labelling. Boxed area shown in higher magnification to visualize labelling. **B:** Negative control, using excess of unlabelled probe, of the same islet as in A in a consecutive section. Islet perimeters are indicated with dashed lines. Scale bar = 50µm.

Figure 4. Apelin mRNA expression in human isolated islets (n= 4) after 48 h of culture in medium supplied with 3.3 mM (LG) or 16.7 mM (HG) glucose (A) or 0 and 6.25 nM dexamethasone (DEX) (B). Apelin mRNA is downregulated by DEX (B), but unaffected by high glucose (A). **p<0.01 vs C.

Figure 5. Immunofluorescence photomicrographs of sections of rat (A-F) and mouse (G-L) pancreas. Double immunostaining for apelin and insulin (A-C and G-I), apelin and glucagon (D-F and J-L). Arrows exemplify coexpression. Apelin is present in both beta cells and alpha cells in both species. Scale bar = 50µm.

Figure 6. Immunofluorescence photomicrographs of sections of porcine (A-C) and feline (D-F) pancreas. Double immunostaining for apelin (A, D) and insulin (B, E); merged in (C, F). Arrows exemplify coexpression. Apelin is primarily localized to beta cells in both species. Scale bar = 50µm.

Figure 7. Insulin secretion from INS-1 (832/13) cells treated with 2.8 or 16.7 mM glucose and apelin-36. Insulin secreted to the media after the one hour static incubation was measured with ELISA. At 16.7 mM glucose addition of 1μM apelin augmented insulin secretion. On the other hand addition of 10nM and 100nM apelin caused a robust reduction of insulin secretion. Results are from 8 experiments run in quadruplicate. *** $p<0.001$ vs 16.7 mM glucose and 0 nM apelin.

Figure 8. Quantification of the ratio of beta cells with apelin expression in db/db-mice and WT mice (A) and in GK-rats and Wistar rats. Both models of type 2 diabetes exhibit higher beta cell apelin expression than the controls. db/db-mice have 40% more beta cells with apelin than WT mice, GK-rats have 74% more beta cells with apelin than Wistar rats. *$p<0.02$, db/db vs wt. ** $p<0.002$, GK vs Wistar.

Fig 1

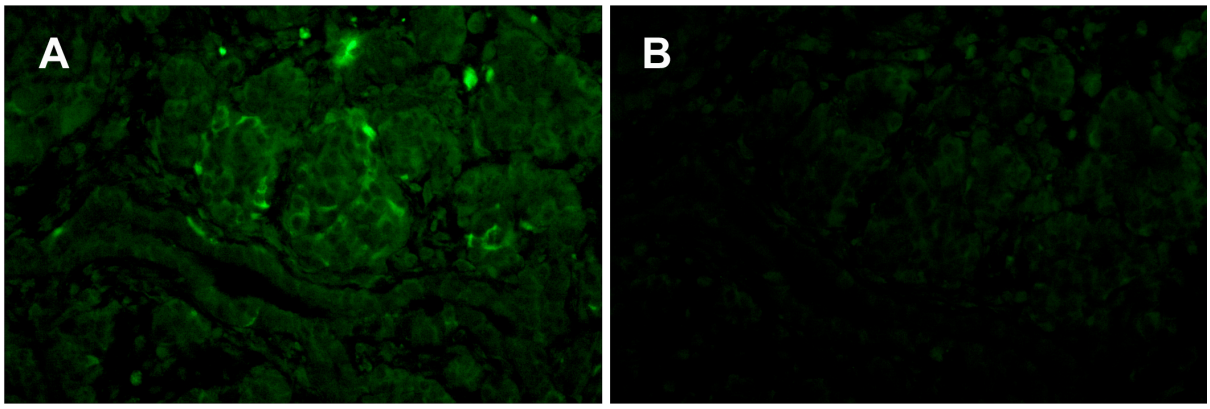


Fig 2

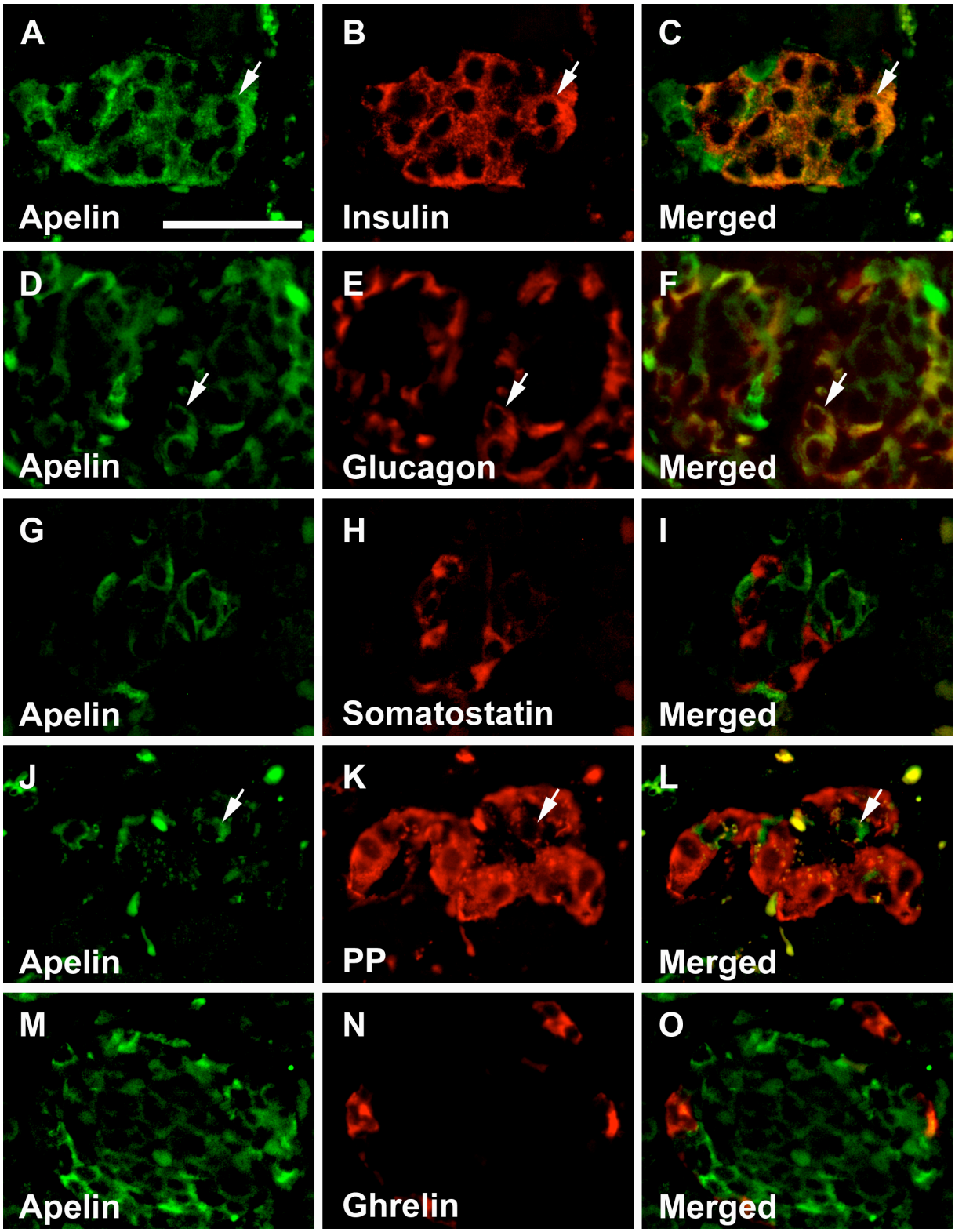

A
Apelin
B
Insulin
C
Merged
D
Apelin
E
Glucagon
F
Merged
G
Apelin
H
Somatostatin
I
Merged
J
Apelin
K
PP
L
Merged
M
Apelin
N
Ghrelin
O
Merged

Fig 3

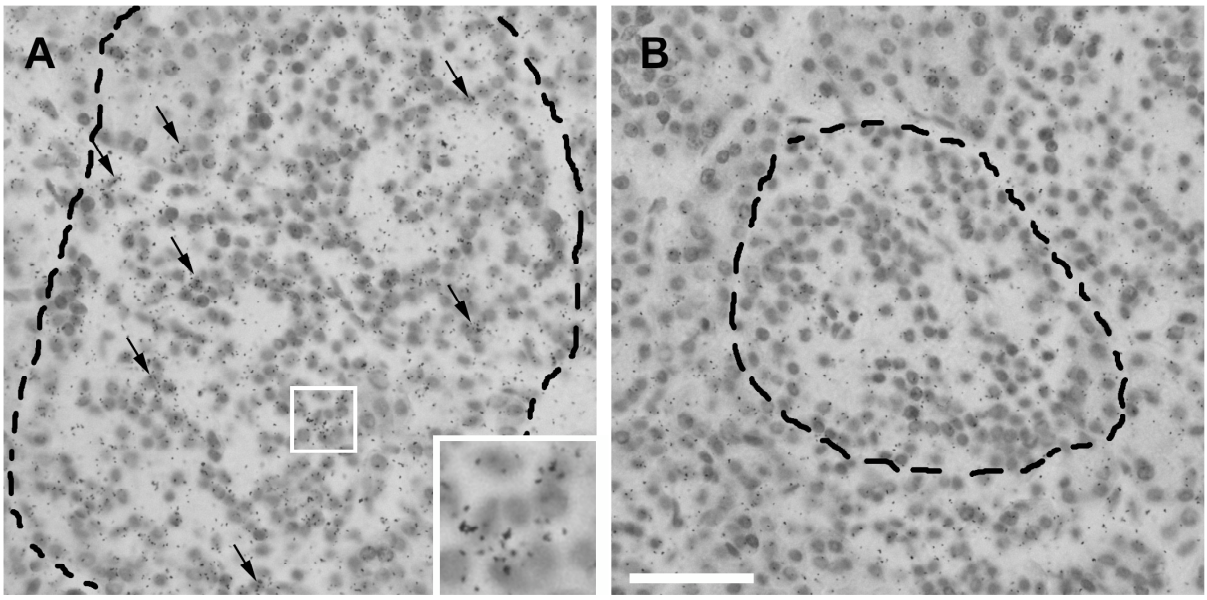


Fig 4

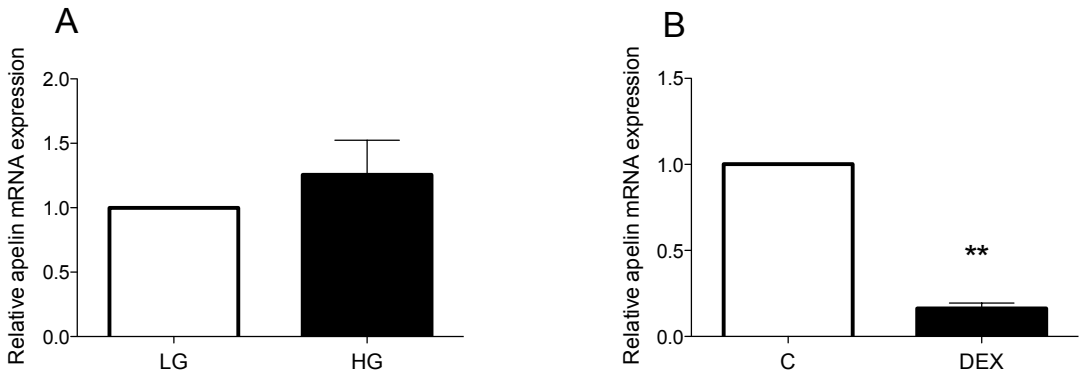

A
Relative apelin mRNA expression
2.0
1.5
1.0
0.5
0.0
LG
HG
B
Relative apelin mRNA expression
1.5
1.0
0.5
0.0
**
C
DEX

Fig 5

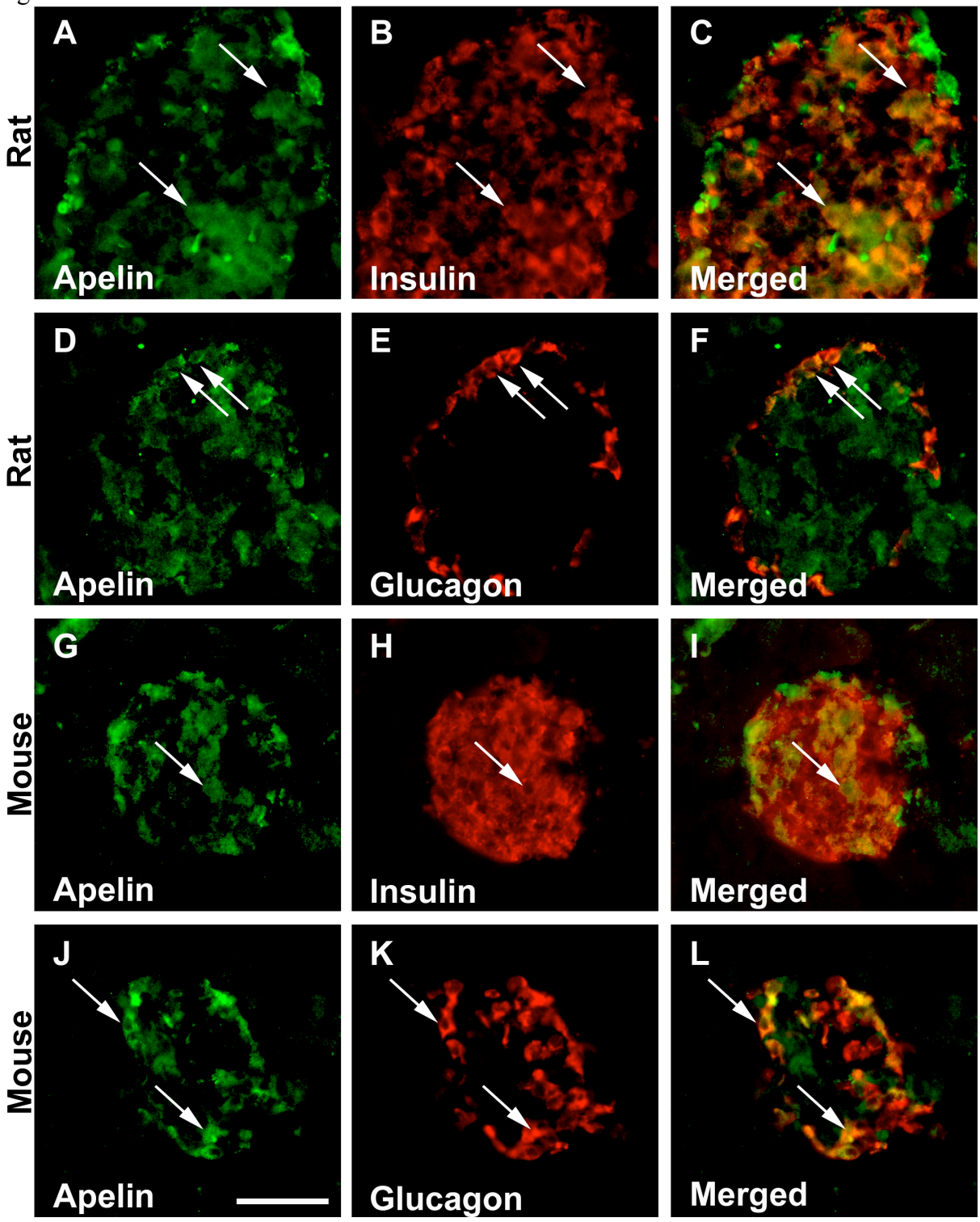

A
B
C
Rat
Apelin
Insulin
Merged
D
E
F
Rat
Apelin
Glucagon
Merged
G
H
I
Mouse
Apelin
Insulin
Merged
J
K
L
Mouse
Apelin
Glucagon
Merged

Fig 6

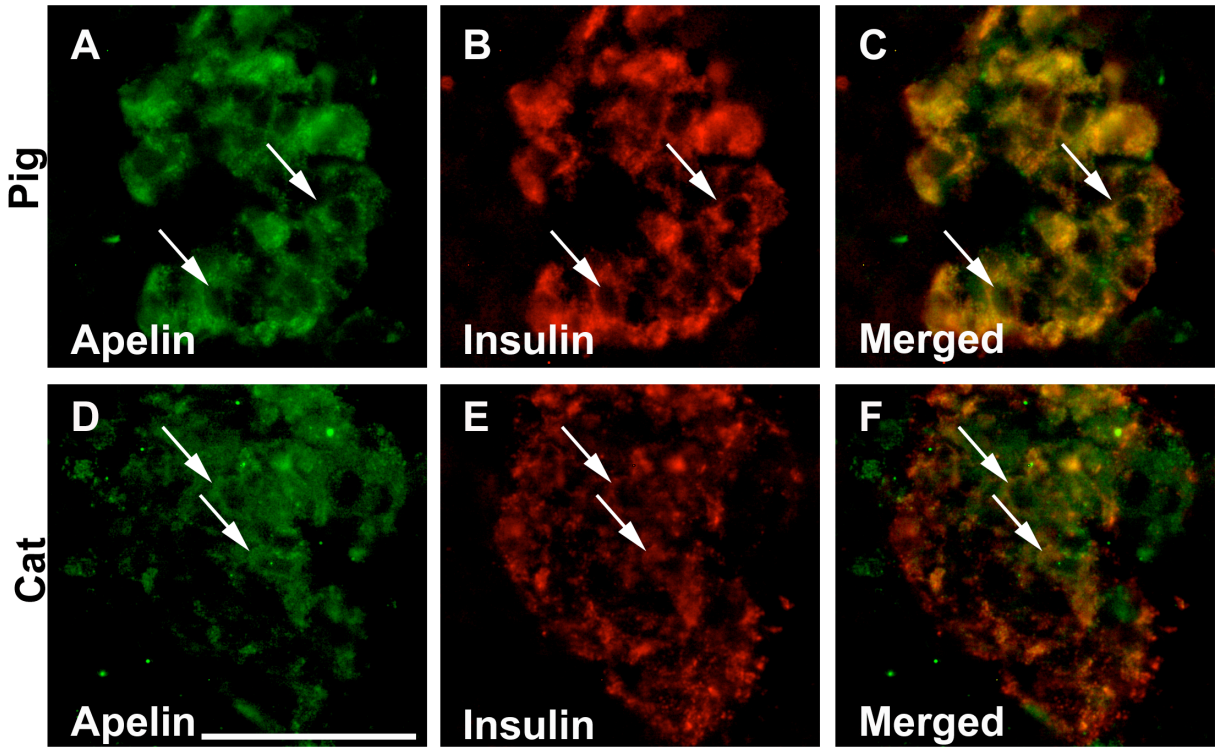

A
B
C
Pig
Apelin
Insulin
Merged
D
E
F
Cat
Apelin
Insulin
Merged

Fig 7

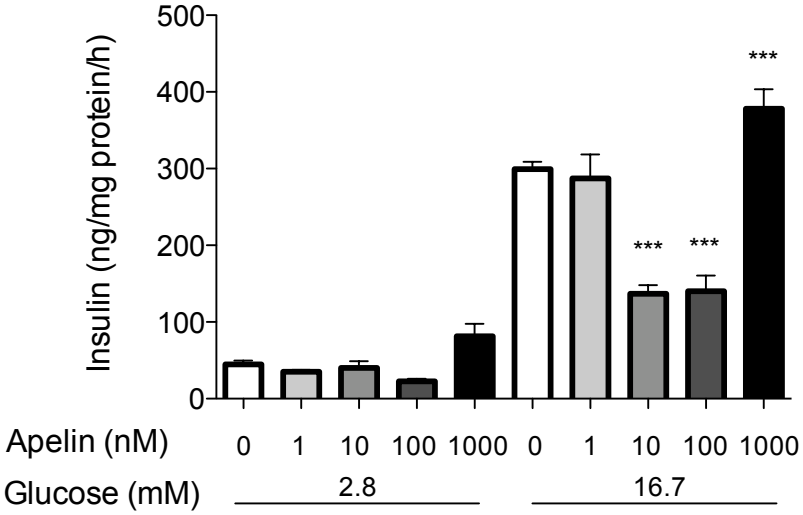
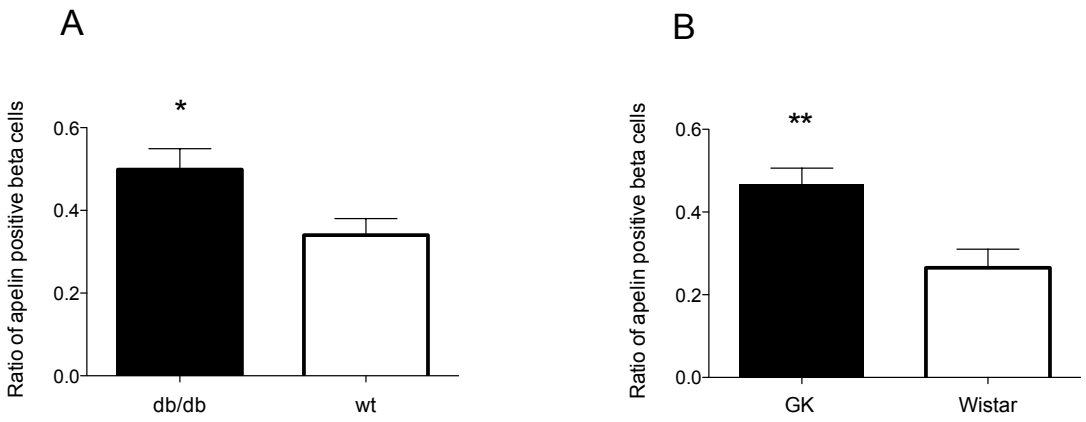

Insulin (ng/mg protein/h)
500
400
300
200
100
0
***
***
***
Apelin (nM)
0 1 10 100 1000 0 1 10 100 1000
Glucose (mM)
2.8
16.7

Fig 8


A
B
Ratio of apelin positive beta cells
0.6
0.4
0.2
0.0
*
db/db
wt
**
GK
Wistar