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The Intestinal Glucose-Apelin Cycle ControlsCarbohydrateAbsorption in Mice.

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Running title: Regulation and role of apelin in mouse intestine.

Abbreviations:

AMPK AMP protein Kinase
APJ apelin receptor
BBM Brush-border membrane
CCK cholecystokinin
GLP glucagon-like peptide
Isc Short-circuit current
PKA protein kinase A
PKC Protein Kinase C
RELM-β resistin-like molecule- β

SGLT-1 Sodium-glucose transporter -1

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Author contribution

C.D, Y.S, D.D, E.W, C.V, B.M,R.D, L.L and S.G participated to the acquisition and analysis of data. A.N-S, P.V, L.S. B and R.D obtained funding and supervised the study. P.V, R.D, I.C-L, B.M, B-O.S and C.D designed experiments, drafted the manuscript and supervised the study.

Competing interest

The authors declare that there is no duality of interest associated with this manuscript.

ABSTRACT

Background & Aims: Glucose is absorbed into intestine cells via the sodium glucose transporter 1 (SGLT-1) and glucose transporter 2 (GLUT2); various peptides and hormones control this process. Apelin is a peptide that regulates glucose homeostasis and is produced by proximal digestive cells; we studied whether glucose modulates apelin secretion by enterocytes and the effects of apelin on intestinal glucose absorption.

Methods: We characterized glucose-related luminal apelin secretion in vivo and ex vivo by mass spectroscopy and immunological techniques. Effects of apelin on ^{14}C-labeled glucose transport were determined in jejunal loops and in mice following apelin gavage. We determined levels of GLUT2 and SGLT-1 proteins and phosphorylation of AMPKα2 by immunoblotting. The net effect of apelin on intestinal glucose transepithelial transport was determined in mice.

Results: Glucose stimulated luminal secretion of pyroglutaminated apelin-13 isoform ([Pyr-1]-apelin-13) in mice small intestine. Apelin increased specific glucose flux through the gastro-epithelial barrier in jejunal loops and in vivo following an oral glucose administration. Conversely, pharmacological apelin blockade in the intestine reduced the increased glycemia that occurs following oral glucose administration.Apelin activity was associated with phosphorylation of AMPKα2 and rapid increase of GLUT2:SGLT-1 protein ratio in the brush-border membrane.

Conclusion: Glucose amplifies its own transport from the intestine lumen to the bloodstream by increasing luminal apelin secretion. In the lumen, active apelin regulates carbohydrate flux

through enterocytes by promoting AMPKα2 phosphorylation and modifying the ratio of SGLT-1:GLUT2. The glucose–apelin cycle might be pharmacologically handled to regulate glucose absorption and assess a better control of glucose homeostasis.

Keywords: Calorie intake; mouse model; diabetes; adipokine, apelin

INTRODUCTION

Apelin is the endogenous ligand for G protein-coupled receptor APJ[1, 2] acting under several molecular forms ([Pyr-1]-apelin-13, apelin-13, -17 and -36) processed from a 77-amino acids precursor[3].The active forms of apelin are present in peripheral tissues including lungs, heart, adipose and pancreas (for reviews see[4, 5]). In the gastrointestinaltract, mRNA apelin-expressing cells were found in rat and mouse stomach, in mouse duodenum, and in human and mouse colon[1,6]. APJ immunostaining has also been described in the epithelium, goblet cells and crypt cell of the small intestine, and in the smooth muscle layer of rat gastrointestinal tract[7]. APJ is also located in the enteric blood vessels[7]. Thus, the apelin/APJ system may have a potential role in the digestive tract.

Recent studies established that apelin is involved in glucose homeostasis[8]. We demonstrated that iv injection of physiological doses of apelin decreased glycemia and stimulated glucose uptake in skeletal muscles of lean and obese insulin-resistant mice[9]. Moreover, apelin-stimulated glucose transport in muscle was dependent of AMPK activation. Similar results were described in cultured C2C12 myotubes by Yue et al.[10]. These authorsalso showed that apelin-deficient mice exhibitdecreased insulin sensitivity[10]. Taken together, such studies support the assumption that apelin plays a physiological role in glucose metabolism and maintenance of insulin sensitivity[8].

We demonstrated that leptin and resistin-β, two adipokines secreted in the gastrointestinal lumen by gastric and intestinal endocrine cells, regulate the activity of the sugar transporters in enterocytes by an AMPK-dependent mechanism[11-13]. The net effect of this regulation ofhexose transporters was an increase of sugar uptake with significant consequences on splanchnic metabolism. Interestingly, the adipokine apelin shares several features with these peptides such as i) the ability of being produced in gastrointestinaltract, ii) an implication in glucose metabolism and iii) the control of insulin sensitivity *via* AMPK [10,

[11].Recent studies brought evidence of a putative regulation of apelin by glucose in different tissues. Indeed, increased amounts of apelin in response to different glucose levels[14, 15, 27] were demonstrated in human endothelial as well as in pancreatic β-cell.

This study was designed to characterize the relationship between apelin and glucose in intestine.We showthat D-glucose promotes specifically [Pyr-1]-apelin-13 secretion in intestine.We further demonstratein vitro and in vivothe capacity of apelin to increase glucose flux from lumen toward the bloodstream by interacting with the APJ receptor present in enterocytes. This effect appears to involve an AMPK-dependant control of SGLT-1 and GLUT2 expression in apical enterocytes membrane by apelin. Moreover, pharmacological inhibition of endogenousapelin action bya selective APJ antagonist resulted in a decrease of glycemia,supporting the existence of a glucose/apelin cyclethat regulates intestinal carbohydrates absorption.

METHODS

AnimalsMale C57BL/6J mice (Centre Elevage Janvier, Le Genest-St-Isle, France) had free access to water and standard food. They were treated in accordance with European Community guidelines concerning care and use of laboratory animals.

NanoLC-MS/MS analysis. The gastric contents were filtrated with a 10kDa membrane and injected on a NanoRS 3500 chromatographic system (Dionex, Amsterdam, The Netherlands) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Five µL of each sample were separated on a 75 µm ID x 15 cm C18 column (Proxeon Biosystems, Odense, Denmark). Peptides were eluted using a 5 to 50% linear gradient of solvent B in 105 min (solvent A was 0.2% formic acid and solvent B was 0.2% formic acid in 80% ACN). Full MS scans were acquired in the Orbitrap on the 300-2000 *m/z* range with the resolution set to a value of 60000. An inclusion list corresponding to several charge states (2+, 3+, 4+) of [Pyr-1]-apelin-13 was used to select these ions for CID fragmentation and the resulting fragment ions were analyzed in the linear ion trap (LTQ). Dynamic exclusion was employed within 60 s to prevent repetitive selection of the same peptide.

Fluorescence immunohistochemical studies and confocal microscopy
Immunohistochemical staining was performed as previously described[16] using anti-APJ polyclonal antibody (Novus Biologicals, 1/100), anti-apelin polyclonal antiserum (Covalab, 1/200) and anti-GLUT2 antibody (Abcam 54460, 1/100). Nuclei were stained with TOPRO-III (Invitrogen, 1/1000). Fluorescence analysis was performed utilizing a LSM510 Confocal Laser Scanning microscope. Samples were visualized with a 25X objective lens (Plan-Apochromat, N.A. 1,4, Oil) and excited using three laser lines (488, 543 and 633 nm). For

APJ and GLUT2 detection, control was achieved using an IgG mouse serum at the same concentration as the antibody. The specificity of apelin immunostaining was tested using primary antisera pre-absorbed with excess amount of homologous antigen ([Pyr1]-Apelin-13, 10^{-6} mol/L). Densitometric quantifications of fluorescence intensity were assessed by Image J software. The results represent the apelin-integrated density – (total area x mean fluorescence of background) of 3 different pictures per mouse and 4 mice per group.

Tissue preparation and short-circuit measurement Mice were 16 h fasted and euthanized. The proximal jejunum was dissected out and adjacent samples mounted in Ussing chambers. The tissues were bathed with Krebs Ringer solution (KRB) with 10 mmol/L glucose at 37°C (pH 7.4) and were gassed with 95% O_2/5% CO_2. Electrogenic ion transport was monitored as previously described [11]. KRB alone (vehicle) or containing apelin (10^{-10} to 10^{-6} mol/L) was added in the mucosal bath 2 min before the 10 mmol/L-glucose challenge. Similar tests were performed with 100 nmol/L apelin incubated overnight at 4°C with 1/100 rabbit polyclonal antibody raised against apelin (Covalab, France).

Transmural hexoses transport The experiments were performed using jejunal sacs from fasted mice. The proximal jejunum was dissected out and rinsed in cold saline solution. Jejunal sacs (4 cm long) were prepared for D-[1-^{14}C] glucose (49.5 mCi/mmol) transport as previously described[11]. The corresponding jejunal sacs were filled with 1 ml of KRB solution without (control) or with 1 nmol/L apelin and containing 0.02 µCi/ml of the isotopic tracer D-[1-^{14}C] glucose (49.5 mCi/mmol) and glucose to obtain a final concentration of 30 mmol/L. Similarly, we studied the paracellular transport with 30 mmol/L mannitol and the isotopic tracer D-[1-^{14}C] mannitol (59 mCi/mmol) at 0.2 µCi/ml.

SGLT-1, GLUT2, AMPKand APJ western blot Fastedanimals were anesthetized and laparotomized for *in situ* experiments. Three jejunal segments (5 cm long) were tied and filled with 3 ml of KRB without (control) or with 1 nmol/L apelin. After 3 min of *in situ* incubation, 3 ml of 60 mmol/L glucose solution were injected in the lumen to obtain a final concentration of 30 mmol/L. After a further 5 min, these sacs were removed and opened along the mesenteric border and the mucosa was scraped off on ice with a glass blade.

For APJ determination, mice were gavaged with water (control) or [Pyr-1]-apelin-13(200 pmol/kg in 100 µl). After 10 minutes, mice were euthanatized and whole intestine was dissected on ice. The total cell protein extracts and the brush-border membranes (BBM) were prepared from the scrapings as previously described[17].Solubilized proteins were resolved by electrophoresis on 10% SDS-PAGE gels and proceeded for immunoblotting. The followingantibodies were used at a 1:1000 dilution: SGLT-1 (AB 1352; Chemicon International); GLUT2, phospho-AMPK-α1/2 (Thr172) and AMPKα 1/2 (sc-9117, sc-33524, sc-25792, respectively; Santa Cruz Biotechnology) and 1:500 for APJ (NLS 64, Novus Biologicals). The intensity of the specific immunoreactive bands was quantified using NIH Image (Scion).

In vivo luminal apelin secretion Fasted C57BL/6J mice were orally loaded by 100 µl of D-glucose solution (0.5 or 1 g/ml) or water (control). After 10 minutes, mice were euthanatized, whole intestine was dissected and the luminal content gently collected and immediately frozen.

Apelin secretion and immunoblotting from everted intestinal sacs Whole proximal intestine was harvested, rinsed, everted and filled with PBS. Then, 1 cm long everted-

intestine sacs were incubated in gassed and warmed PBS with or without glucose (5 and 30 mmol/L). Fifty µl of media were collected at time 5, 30 and 60 min of incubation and immediately frozen in liquid nitrogen before a column-filtration step (Amicon Ultra, 10,000 MWCO, Millipore) and measure of apelin concentration. To normalize results obtained, each sac was collected for protein quantification. The same experiments were done to determinate apelin-77 content in jejunal mucosa by western blotting after 60 minutes with (30 mmol/L) or without glucose in KH buffer incubation. Solubilized proteins were resolved by electrophoresis on 10% SDS-PAGE gels. Apelin-77 (Abcam 59469) and β-actin (Cell Signaling Technology 13E5) antibodies were used at a 1:500 dilution. The intensity of the specific immunoreactive bands was quantified using NIH Image (Scion).

Apelin's effect on oral glucose load Gavages of fasted mice with a D-glucose solution (3 g/kg of body weight) were performed. Glucose load was preceded (10 min) by a PBS (control) or a [Pyr-1]-apelin-13 (200 pmol/kg in 100 µl) gavage. Ten minutes later, mice were anesthetized and the blood was harvested from hepato-portal vein for glucose (RTU kit Roche) and apelin concentration determination. Same experiments were performed in presence or absence of APJ antagonist (1 µmol/kg in 200 µl, 10 min before glucose load).

APJ-mediated β-arrestin 2 recruitment measured by BRET assay Fasted mice were gavaged with apelin (200 pmol/kg in 100 µl) or APJ antagonist (1 µmol/kg in 200µl) and the luminal content of proximal intestine was collected 10 minutes later and immediately frozen in liquid nitrogen. After a step of filtration and purification, apelin and antagonist's activities of luminal content of PBS-, apelin- or APJ antagonist-gavaged mice were assessed on β-arrestin 2 recruitment to APJ by BRET. HEK-293T cells were transfected with the human

apelin receptor tagged on its C-terminus with the BRET donor Renilla luciferase and β-arrestin2 double tagged with the yellow fluorescent protein (YFP) at the N- and C-terminus. BRET approach was assayed as previously described[18]. Intrinsic activities of filtered luminal contents were measured by adding samples 5 min after the Rluc substrate coelenterazine. To determine ifthe antagonist was still active after gavage, luminal content was added 1 min after the luciferase substrate and exogenous [Pyr1]-apelin-13 was added 5 min before reading.

Apelin assay Apelin was quantified with the non-selective apelin-12 EIA kit (Phoenix Pharmaceuticals, Belmont, CA). Before dosage, luminal content, plasma and conditioned medium were filtrated and concentrated by column (Amicon Ultra, 10,000MWCO, Millipore).

qPCR experiments Jejunal loops were incubated during 24h in warmed oxygenated KRB containing [Pyr1]-apelin-13 (10^{-6} mol/L) or APJ-antagonist (5.10^{-6} mol/L) and/or D-glucose (30 mmol/L). After treatment, loops were rinsed in KR buffer and immediately liquid nitrogen-frozen for GLUT2 and SGLT-1 mRNA quantification as previously described.[8].

Chemicals [Pyr1]-Apelin-13 was purchased from Bachem (Switzerland), D-[1-^{14}C] mannitol was from GE Healthcare Amersham Biosciences, (les Ulis, France) and D-[1-^{14}C] glucose from Perkin Elmer, (Boston USA). All other chemical reagents were purchased from Sigma (St. Louis, MO). Apelin antagonist (C-14-C dicyclic[19]) was purchased from Polypeptide (Strasbourg, France).

Statistical analysis All results were expressed as means ± SEM. One-way ANOVA with Turkey-Kramer multiple comparisons posthoc-test was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Significance was set at $p<0.05$.

RESULTS

Glucose increases luminal secretion of apelin in vitro and in vivo When administrated by gavage to mouse, exogenous glucose promotes apelin luminal secretion. Indeed, 10 minutes after an oral load with high glucose solutions (50 or 100mg in 100 µl of water), luminal apelin amount measured in the collected luminal material raised by twofold (Fig. 1a). This regulation is glucose-specific and independent of osmolarity since the same concentration of mannitol did not induce apelin secretion (supplemental Fig.S1). Consequently, as shown in figure 1b and c, apelin contained in jejunal cell cytoplasm (red)was partially depleted when glucose was orally-given, leading to a significant decrease of immunoreactivity. No staining was observed when the same experiments were performed with preabsorbed immune serum (Fig. 1b, bottom panel).Taken together, these results suggest that apelin stored in the epithelial cells (supplemental fig. S2) has been released in the lumenin response to glucose. To confirm this hypothesis, we further quantified apelin secretion ex vivo on everted intestine loops from mouse duodenum and jejunum (Fig. 1e and f). Both tissues exhibit the same kinetic apelin secretion profile characterized by a dramatic increase of apelin concentration in the medium 30 minutes after incubation of everted loops with 5 or 30 mmol/L glucose solutions. After 60 minutes, similar increase was observed suggesting that maximal release of apelin was reached alreadyafter 30 minutes.Moreover, western blottings showing intracellular form of apelin (dimer of apelin-77; 16kDa) in jejunal-everted loop incubated 60 minutes with 30 mmol/L of glucose corroborate these results and reinforce a glucose-dependent apelin release (Fig. 1d).Then, in order to determine ifimmunoreactive quantification of apelin was specific of one isoform, we performed mass-spectrometry(MS) analysisof gastric secretions collected from PBS-, apelin- or glucose-gavaged mice. As[Pyr-1]-apelin-13(resulting from a pyroglutamination of apelin-13) is the most stable isoform in aqueous phases, comparisonswere done witha control profile processed with synthetic [Pyr-1]-apelin-13 alone

(data not shown and [20]) or addedin PBS-treated mice luminal content(figure 2a). Figure 2ashows that after purification and filtration steps of gastric secretions, spiked synthetic [Pyr-1]-apelin-13 can be recovered by this technique. Figures 2b and 2e (middle panel) show that after [Pyr-1]-apelin-13 gavage, the same peptide is recovered in intestinal lumen of mice after 10 minin contrast to PBSorally-load mice(figure 2d). Moreover, after glucose load (1g/ml), [Pyr-1]-apelin-13was also detected in gastric secretions (figure 2c and 2e; lower panel) in the same extend. FurtherMS experiments and analysis based on ion signal extraction indicated that other apelin isoforms were not recovered in glucose-load mice lumen secretions when compared to their published MS profile (supplemental figure S3).

Exogenous apelin binds APJ receptor present in brush-border membrane of enterocyteand controls abundance of glucose transporters in BBM. As apelin secretion appears to be controlled by glucose, we investigated the reciprocal role of [Pyr-1]-apelin-13 on enterocyte glucose pathways. We first measured the presence of apelin receptor on small intestine. Fluorescent immunohistological studies were performed on mouse jejunal mucosa sections showing that apelin receptor (APJ) is expressed in villi associated primarily with the cell membrane (Fig. 3a). No staining was observed when the same experiments were performed with non-specific IgG antibody. Moreover, immunoreactive signal corresponding to apelin receptor APJ was found by western blotting in total proteins (fig 3b) and brush-border membranes (fig 3c) from mice enterocytes. Significant signal was found in BBM in basal condition indicating a constitutive expression of APJ receptor. However, immunoreactive signal was 3.9-fold decreased ($p=0.013$) when [Pyr-1]-apelin-13 was given to the mice (Fig.3c) suggesting effective activation of APJ receptor by exogenous [Pyr-1]-apelin-13 and consequently APJ internalization.

Intestinal glucose physiology is characterized by post-translational regulationofglucose transporter abundance in BBM. We thus examined ex vivo whether apelin modifies glucose transporters amount and activityon jejunal loops. As expected, glucose (10 mmol/l) induced a 2.4-fold increase of SGLT-1 amounts in BBM compared to controls (Fig.4a). Apelin (1 nmol/l) alone significantly reduced the basal level of SGLT-1 ($p<0.01$) and markedly prevented glucose-increase in SGLT-1 abundance ($p<0.001$) when injected into the loop 3 minutes before glucose (Fig. 4a).

The effect of apelin on SGLT-1 activity was then studied using Ussing chamberon mice jejunum isolated. As previously described[11, 12, 21], the addition of 10 mmol/L D-glucose to the mucosal bath of Ussing chamber induced a rapid and marked increase in Isc (vs. basal conditions), reaching a plateau after 3-4 minutes. Addition of apelin in mucosal compartment 3 minutes before glucose challenge markedly reduced the glucose-induced Isc (ΔIsc). As depicted in figure 4b, the inhibition of ΔIsc was dose-dependentwith a maximal inhibition of 0.1 µmol/L and an IC_{50} of 0.1 nmol/L. Overnight incubation with apelin antibody totally blocked the inhibitory effect of the peptide (Fig. 4c). Since SGLT-1 expression is balanced byGLUT2 expression [14], the effect of apelin on GLUT2 was studied. Apelin induces a significant increase in the abundance of GLUT2 in BBM ($p<0.05$) (Fig. 4d). Glucose alone induces a 2.1-fold increase in apical GLUT2 protein andapelin co-incubated with glucose resulted in a significant increase in immunoreactive GLUT2 over the value of glucose or apelin alone indicating an amplification mechanism or additive effects via distinct pathways (Fig. 4d).

Since AMPK is a key-regulator of glucose transporter in enterocyte, we measured the effect of apelin on the phosphorylation of AMPKα2 subunit. Apelin alone significantly stimulated AMPKα2 phosphorylation (Fig. 4e). This effect was less marked when compared with glucose alone but when apelin was injected in the jejunal loop together with glucose, the

phosphorylation of AMPKα2 was significantly enhanced showing an additive effect of apelin and glucose (Fig. 4e).

Apelin stimulates transepithelial transport of ^{14}C-glucosethrough GLUT2 regulation Since AMPKα2 activation is associated with glucose flux control in enterocyte[13], we investigated[Pyr-1]-apelin-13 effect on transmural hexoses transport (Fig. 5). As shown in Fig. 5a, [Pyr-1]-apelin-13 (1 nmol/L) significantly increased 30 mmol/L glucose uptake in the isolated mice jejunum. This effect was fast (2 min after apelin addition) and glucose specific. Indeed, no effect on 10 mmol/L mannitol uptakewas observed indicating that the increased transport of glucose induced by apelin was unlikely to be caused by changes in paracellular permeability (Fig. 5b). To better understand the apelin's pathways allowing this glucose flux, we performed the same experiment in presence of phloretin, a GLUT transporter selective inhibitor. The results show that the transepithelial glucose transport stimulated by apelin is significantly decreased to control value in presence of the inhibitor suggesting that GLUT2 translocation is necessary for apelin's effect (Fig. 5c).

Orally-given apelinincreases intestinal transepithelial glucosetransport from lumen to bloodstreamin mice

The net effect of oral apelin on glucose absorption was further investigated. For that purpose, the consequence of an oral administration of [Pyr-1]-apelin-13 on hepato-portal glucose concentration was studied duringan oral glucose load in fasted mice. Orally-given apelin dramatically increasedglucose concentration in hepato-portal vein15 minutes after glucose load (Fig. 6a). Then, in order to demonstrate thephysiological effect of endogenous apelin production on intestine glucose uptake,we inhibited theglucose/apelin cycle by using a specific APJ antagonist[19]orally-given 10 minutes before the glucose load.Data presented in

figure 6b show that 1 μmol/kg antagonist promoted a marked reduction of plasmatic glucose 10 and 20 minutes after the glucose load. This result clearly indicates that apelinphysiologically promotes carbohydrates absorption.Finally, to evaluate the long-term effect of both apelin and APJ-antagonist, we studied the consequence of a 24h-treatment on SGLT-1 and GLUT2 mRNA level (Fig. 6c). As expected, GLUT2 was significantly increased while SGLT-1 mRNA level was decreased by 30 mmol/l glucose. Apelin had the same effect than glucose on carbohydrate transporters mRNA expression.Such effects were blunted by APJ-antagonist treatment.

Exogenous apelin given by gavage remains active in the proximal intestine

To know if exogenous [Pyr-1]-apelin-13 and APJ-antagonist orally-given to mice are still active when they reach proximal intestine, the luminal content was collected 15 minutes after gavage, purified, filtered and tested for APJ-inducing β-arrestin 2 recruitment. Control of cell transfection as well as BRET experiment validity obtained by increasing doses of apelin are presented in supplemental data (supplemental figure S5). Luminal content collected from [Pyr-1]-apelin-13 orally-loaded mice displayed a significant rise in BRET signal compared to PBS-stuffed mice, indicating that orally-loaded apelin is active and induces arrestin recruitment to the membrane (Fig. 7a). Conversely, when APJ-antagonist was given by gavage to mice, the resulting luminal content did not modify basal APJ activity by BRET. Finally, the efficiency of the APJ-antagonist collected after gavage was studied on apelin-induced BRET signal (Fig. 7b and c). As expected, exogenous [Pyr-1]-apelin-13 added to cells (10^{-7} mol/L and 10^{-6} mol/L, Fig. 7b and c respectively) induced a significant increase in BRET signal. This effect was totally blunted by lumen content from APJ-antagonist orally-loaded mice when 10^{-7} mol/L exogenous apelin was used (Fig. 7b.) but not for a higher dose of the peptide (10^{-6} mol/L, Fig. 7c). These results demonstrate that exogenous [Pyr-1]-apelin-

13and APJ-antagonist remain efficient when they reach glucose absorption zones of the intestine, i.e. duodenum and jejunum.

DISCUSSION

This study demonstrates the presence of a regulatory intestinal loop between apelin and glucose leading to a rapid regulation of intestinal glucose absorption. In order to insurebalanced glucose absorption during or after a meal, the activity of sugar transporters in the enterocytes appears highly regulated. Indeed, glucose itself is able to promote its own transit through the intestinal barrier towards bloodstream by a fine regulation of SGLT-1 and GLUT2 abundance in the BBM[22]. Recent studies describedotheractors of glucose absorption implicating hormones such as CCK[23], angiotensin II[24], insulin[25], leptin[11], RELM-β[12] or GLP1/2[26]. Apelinshares characteristics features with these hormones, particularly with leptin, since both are increased with obesity, target the intracellular AMPK and are closely related to glucose homeostasis and insulin sensitivity through their action on skeletal muscle and adipose tissue[9, 27]. In this study, we demonstrate by different approaches that glucose specifically enhances [Pyr-1]-apelin-13 secretion from intestine cells into the lumenwithout modifications in plasma apelin (supplemental fig. S4). This is in line with our previous demonstration that luminal secretion of gut leptin was observed without any modification of blood leptin levels in rat [13].Eventhough apelin and glucose have been tightly associated in several studies, the direct effect of glucose on a putative apelin secretion has been poorly described. Yamagata et al. have recently shown that human endothelial cultured cells secrete apelin in response to glucose andRingstrom et al. describeda slight increase of mRNA apelin in isolated human pancreatic islets after glucose stimulation[15, 28]. In our study, although concentrations of glucose used ex vivo(30 mmol/L) are physiologically relevant and comparable to other studies[12, 13], in vivoexperiments required higher doses of glucose (up to 100mg/100µl) to ensure that a significant amount of glucose reaches small intestine few minutes after oral load.

Apelin secretion in the lumen in response to sugar is in line with previous demonstration of luminal leptin secretion in the intestine after fructose load[13]. The question arises of bioavailability of peptides in the lumen characterized by its acidic pH and proteolytic enzymes activity. Interestingly, orally-given apelin remains active as shown by APJ activation and BRET studies. Leptin was shown to besecreted in the lumen together with a soluble receptor allowinga protection from degradation by proteolytic enzymes[29].It could be hypothesized that such an associated protein could exist for apelin and further consideration to propose pharmacological modulations of luminal free/bound apelin concentrations are needed.

This glucose-induced luminal apelin secretion led us to investigate the functional effect of [Pyr-1]-apelin-13 on glucose absorption. We showed by different approaches that physiological concentration of apelinenhances glucose transmural transport from lumen to the bloodstream.This effect is specific for glucose and not associated with modifications of enterocytes tight-junction's permeability since mannitol is not translocated from the lumen to the serosal side in presence of apelin. Our results also show that, consistently with previous works in muscle and adipose tissue[9, 30], apelin can also activate AMPK in enterocytes. AMPK is an important glucose sensor in enterocyte[13, 31]and its activation mustinitiate apelin's effects on glucose transportersbalance. Indeed, apelin induces ex vivodecrease of SGLT-1 in BBM whereas GLUT2 expression wasincreased. SGLT-1 is a high-affinity low-capacity glucose carrier constitutively expressed in the BBM of epithelial cell lining the small intestine lumen. SGLT-1 is responsible for luminal glucose uptake against concentration gradient using a membrane potential generated by the sodium pump[32]. Conversely, when glucose concentration increases in the small intestine lumen, mechanisms including PKA, PKCβII and AMPKα2 activation are rapidly activated to traffic GLUT2 into the BBM.The fact that [Pyr-1]-apelin-13 controls SGLT-1's activity and further triggers a switch in glucose transporters

SGLT-1/GLUT2 ratio, activities and gene expression, givesa new insight into control of physiological sugar absorption by apelin.It has been reported[16] that GLUT2 is over-expressed in enterocytes during metabolic diseases which could participate to post-absorptive elevated glycemia. Thus, as showed here, pharmacological approach consisting in the blockade of theglucose/apelin cycle by using oral APJ antagonistcould beused to lower glucose absorption.

In summary, the present data show the involvement of the apelinergic system in mechanisms controlling the intestinal absorption of glucose. As we recently described a putative role for apelin in glucose uptake in muscle and adipose tissue, it could be hypothesized that apelin firstly acts by enhancing intestinal glucose uptake from digested sugars in order to secondarily furnish energetic substrate to apelin-activated tissues. Consequently, intestinal apelin regulation by pharmacological agents such as APJ antagonistscould allow a better controlof blood sugaramounts after a meal. This process could be compared to the effect of lipase inhibitorsthat areclinically used to avoid lipid absorption.Finally, the present study brings new paradigm on luminal secretion, bioavailability and efficiency of gut peptides and paves the way for future utilization of apelin or apelin-antagonist by oral route.

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LEGENDS TO FIGURES

Figure 1: D-glucose triggers intestinal apelin secretion. **a)**In vivo apelin secretion measured in luminal fluid collected from proximal part of intestine after gavage. n=5; * $p<0.05$ vs. no glucose. **b)**and **c)**Confocal micrographs (x25 top line and x50 middle line) of jejunum sections from mice treated or not with oral glucose load (50 and 100 mg in 100 µl) and stained with anti-apelin immune serum. Immune serum preincubated with an excess of apelin was used as control (bottom line). The white bars represent 100 µm. **c)** Densitometric quantification of apelin immunoreactivity in jejunum. n=4; * $p<0.05$, **p<0.01 vs. no glucose. **d)** Representative immunoblots of intracellular apelin-77 from mice jejunum sacs treated with 30 mmol/L glucose. Data of densitometric analysis are expressed as relative protein levels (β-actin as control) n=3; * $p<0.01$.**e)** and **f)** Kinetics of apelin secretion by duodenum or jejunum evertedsacs in medium without (0) or withglucose (5 or 30 mmol/L). n=5; * $p<0.05$.

Figure 2: D-glucose induces in vivo [Pyr-1]-apelin-13 isoform luminal secretion. Extracted ion chromatograms of the pQRPRLSHKGPMPF sequence of [Pyr-1]-apelin-13 in gastric secretions of mice gavaged with PBS plus 2 picomoles of synthetic [Pyr-1]-apelin-13 added before MS analysis **(a)** synthetic [Pyr-1]-apelin-13**(b)**, glucose**(c)** and in gastric secretion of mice treated with PBS **(d)**. **e)** Manually annotated MS/MS fragmentation spectra of the pQRPRLSHKGPMPF peptide obtained from experiments **a** (upper panel), **b** (middle panel) and **c** (lower panel). **f)** Sequence of [Pyr-1]-apelin-13.

Figure 3: Presence of APJ in enterocytes and activation by apelina) Jejunum sections from mice stained with anti-APJ antibody, Topro III or both. Pictures are representative of 4 mice. Control was achieved with non-specific IgG antibodies. Photomicrographs are 50x

magnification, the white bar represents 100 μm. **b)** and **c)** Representative immunoblots of APJ and β-actin in jejunum from mice orally-loaded by [pyr1]-apelin-13 (200pmol/kg) (A1, A2, A3) or PBS (C1, C2, C3). Protein detection was achieved from whole jejunum **(b)** or from BBM purified material **(c)**. Data of densitometric analysis using β-actin as control. * $p<0.05$

Figure 4: Luminal apelin promotes AMPK phosphorylation and controls SGLT-1 and GLUT2 presence in enterocyte BBM a) Representative immunoblots of SGLT-1 from mice jejunum sacs treated with or without [pyr1]-apelin-13 in association or not with 30 mmol/L glucose. Data of densitometric analysis using β-actin as control. * $p<0.05$ **,$p<0.01$ and *** $p<0.001$ vs. NaCl. **b)**Effect of luminal [pyr1]-apelin-13on glucose-induced Isc. Electrogenic sodium transport was followed in mouse jejunum in Ussing chamber as an index of active glucose transport by SGLT-1.[pyr1]-apelin-13was added in bath 2 min before10 mmol/l glucose.**c)**Effect of 10 nmol/l [pyr1]-apelin-13 on glucose-induced Isc.after an overnight incubation with an antibody against apelin (Apelin+Ab.) n = 5-7 tissues studied. * $p<0.05$.**d)** Representative immunoblots of GLUT2 proteins in extracts from jejunum mucosa treated with luminal apelin with or without 30 mmol/L glucose. Densitometric analysis of the blots using β-actin as control. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs. NaCl. **e)** Effect of apelin on phosphorylation of AMPKα2 in the jejunum. Phosphorylated AMPKα to total AMPK was used for densitometric analysis. * $p<0.05$ and ** $p<0.01$ vs. NaCl.

Figure 5 Apelin increases transmural transport of D-glucose in jejunal sacthrough GLUT2 control a) Kinetic of transmural transport of glucose was performed in jejunal sacs from mice. Intestinal sacs were incubated during 15 min with 1 nmol/L apelin (●) or vehicle (□) in KRB containing glucose (30 mmol/L) and D-[1-^{14}C] glucose. Data are representative of four experiments, * $p < 0.05$ vs. control. **b)** Similar experiment with 30 mmol/L mannitol. * p

< 0.05 vs. control. **c)** Effect of GLUT2 inhibitor phloretin (1 mmol/L) on apelin-induced glucose transportin jejunal sacs from four mice. * $p < 0.05$ vs. NaCl.

Figure 6:In vivo effect of[Pyr-1]-apelin-13 on intestinal glucose absorptiona) Apelin (200 pmol/kg in 100 µl (black bar) or PBS (100 µl, white bar) was administrated to mice by gavage 10 min before oral glucose load (3 g/kg). Ten minutes after the glucose load, blood was collected from hepato-portal vein for glucose determination. Data are given as mean ± SEM (n=8) per group. ** p<0.01 vs. PBS.**b)**Glycemia after oral glucose load (3g/kg) performed without (white) or with (black) APJ antagonist (1 µmol/kg body weight, 200 µl) given orally 10 minutes before glucose load. ** $p < 0.01$ vs. PBS. **c)** GLUT2 and SGLT-1 mRNA expression in everted jejunal loops treated by [pyr1]-apelin-13 (10^{-6} mol/L), APJ-antagonist (5.10^{-6}mol/L) and/or glucose (30 mmol/L) during 24h. Results are the mean ± SEM of 5 mice. * p <0.05 vs. Ct; ** p <0.01 vs. Ct; # p <0.05 vs. glucose.

Figure 7:Orally-given [Pyr-1]-apelin-13 and APJ-antagonist activityon APJ internalization a) Effect of luminal contents on net BRET signal in transfected cell. Content of proximal intestine was collected 10 min after PBS, apelin or APJ-antagonist mouse gavage and filtered and purified by column (see Methods). n=4-5 mice per group. ** p <0.01 vs. PBS. **b)** and **c)** Effect of luminal content collected from mice staffed with PBS or APJ-antagonist on exogenous synthetic [pyr1]-apelin-13 (10^{-7} mol/L and 10^{-6} mol/L respectively)-mediating β-arrestin 2 recruitment to APJ measured by BRET. n=5 mice per group. ** p <0.01 vs. [pyr1]-apelin-13 alone.

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FIGURES

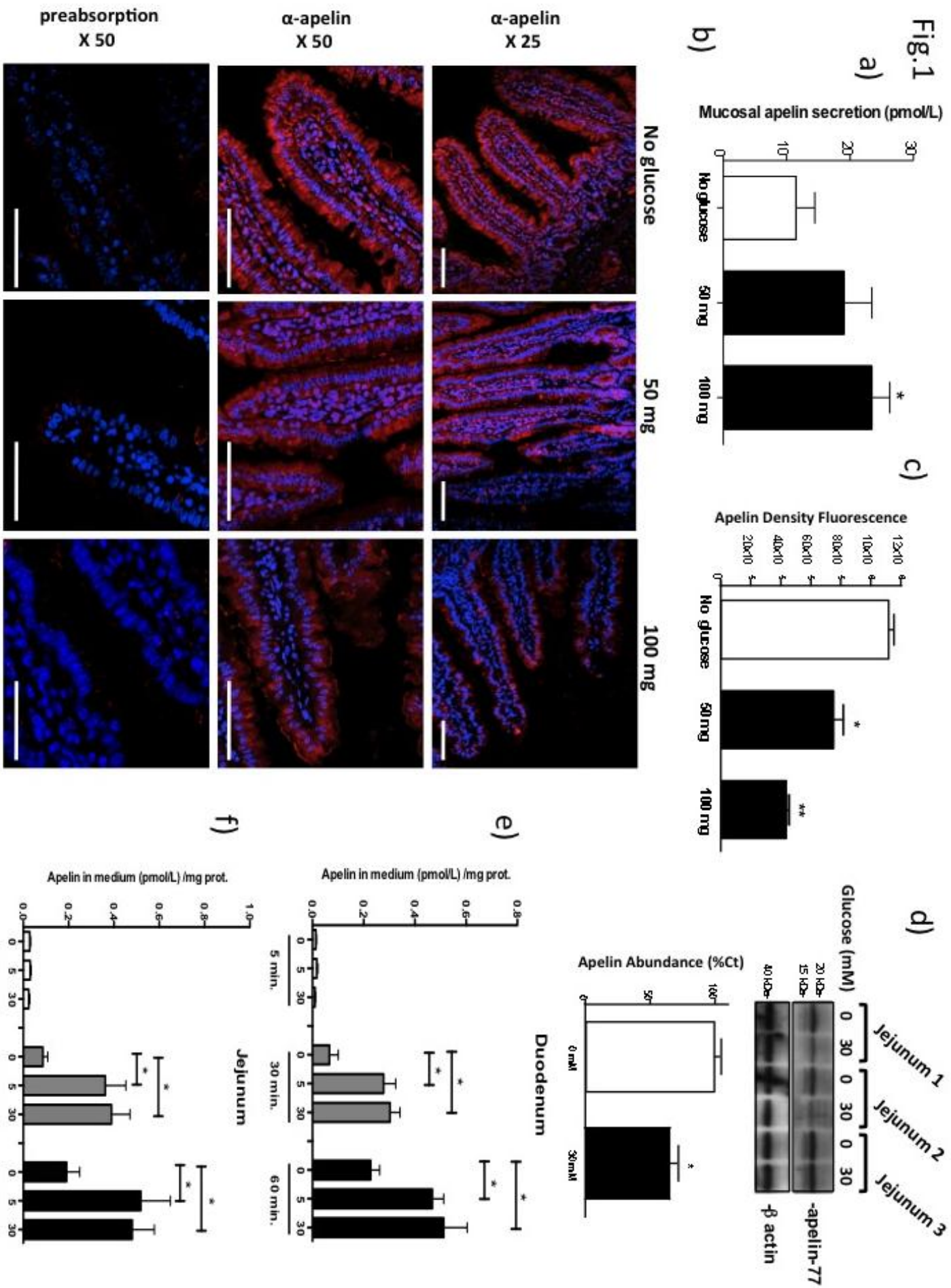


Fig.1
a)
Mucosal apelin secretion (pmol/L)
No glucose
50 mg
100 mg
b)
α-apelin X 25
α-apelin X 50
preabsorption X 50
No glucose
50 mg
100 mg
c)
Apelin Density Fluorescence
d)
Glucose (mM)
Jejunum 1
Jejunum 2
Jejunum 3
-apelin-77
-β actin
Apelin Abundance (%Ct)
0mM
30mM
e)
Apelin in medium (pmol/L) /mg prot.
Duodenum
5 min.
30 min.
60 min.
f)
Jejunum

Fig.2

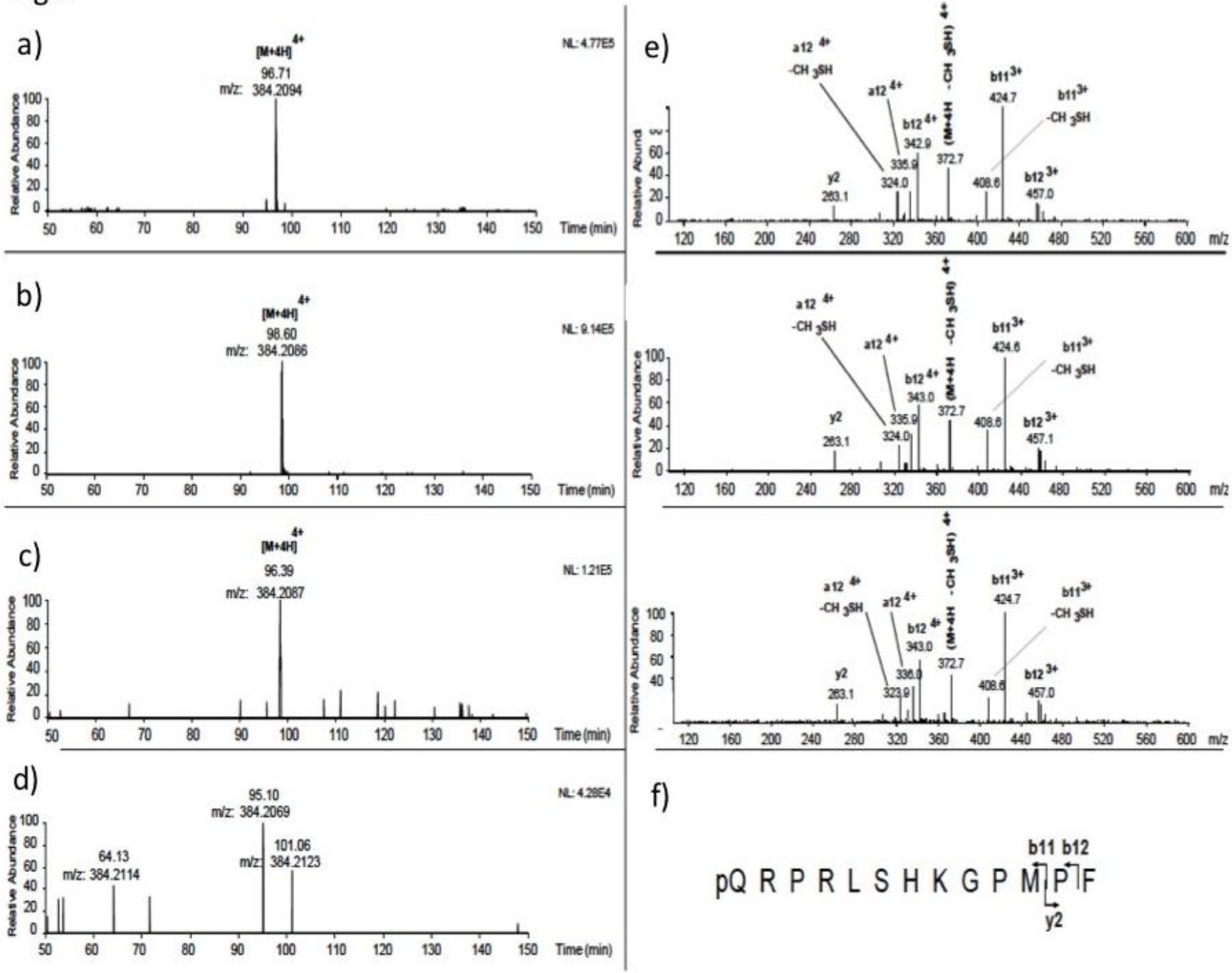

a)
[M+4H] 4+
96.71
m/z: 384.2094
NL: 4.77E5
b)
[M+4H] 4+
98.60
m/z: 384.2086
NL: 9.14E5
c)
[M+4H] 4+
96.39
m/z: 384.2087
NL: 1.21E5
d)
95.10
m/z: 384.2069
64.13
m/z: 384.2114
101.06
m/z: 384.2123
NL: 4.28E4
Relative Abundance
Time (min)
e)
a12 4+ -CH3SH
a12 4+
b12 4+
(M+4H -CH3SH) 4+
b11 3+
b11 3+ -CH3SH
b12 3+
y2
263.1
324.0
335.9
342.9
343.0
336.0
323.9
372.7
408.6
424.7
424.6
457.0
457.1
m/z
f)
b11 b12
pQ R P R L S H K G P M P F
y2

Fig.3

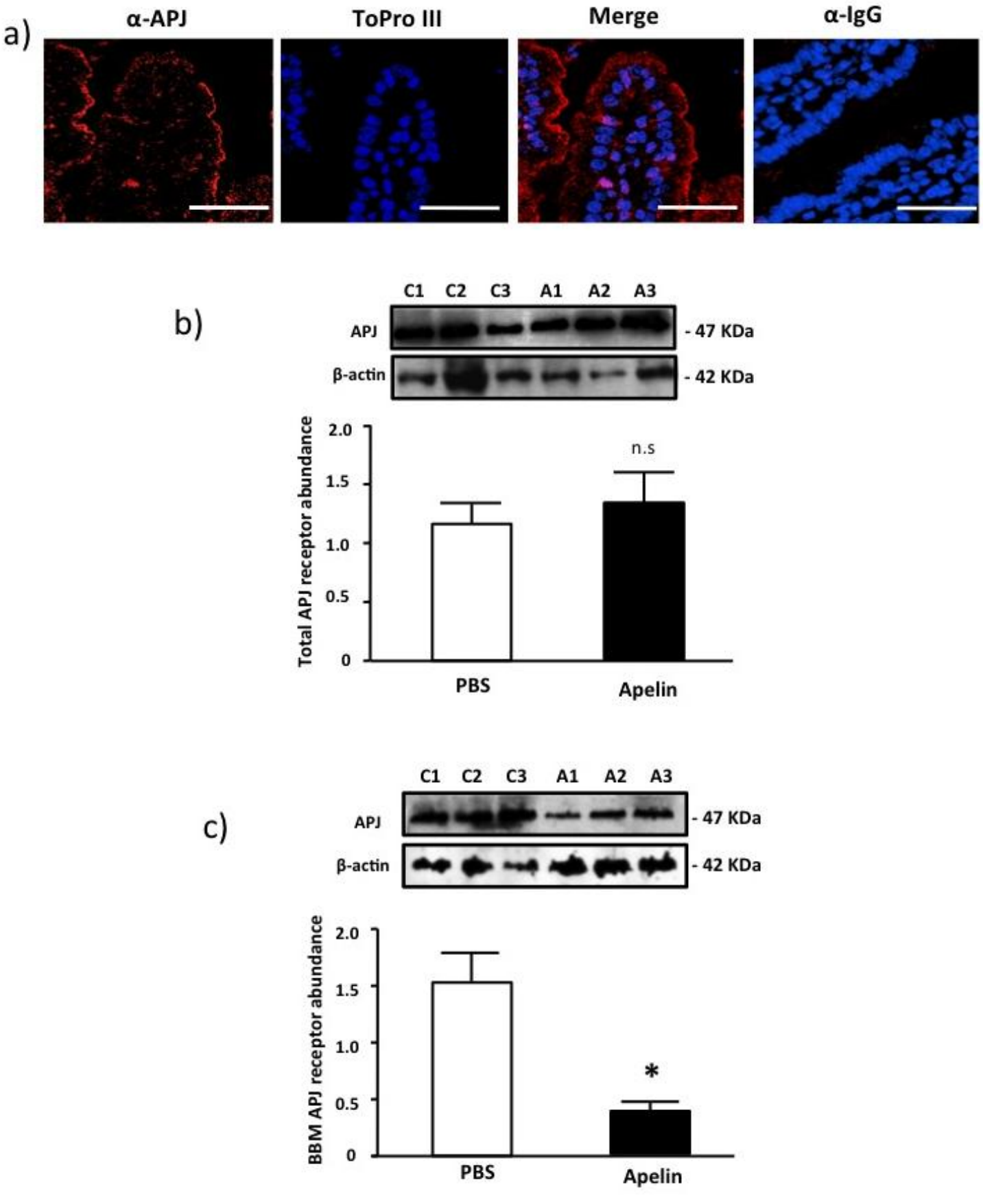
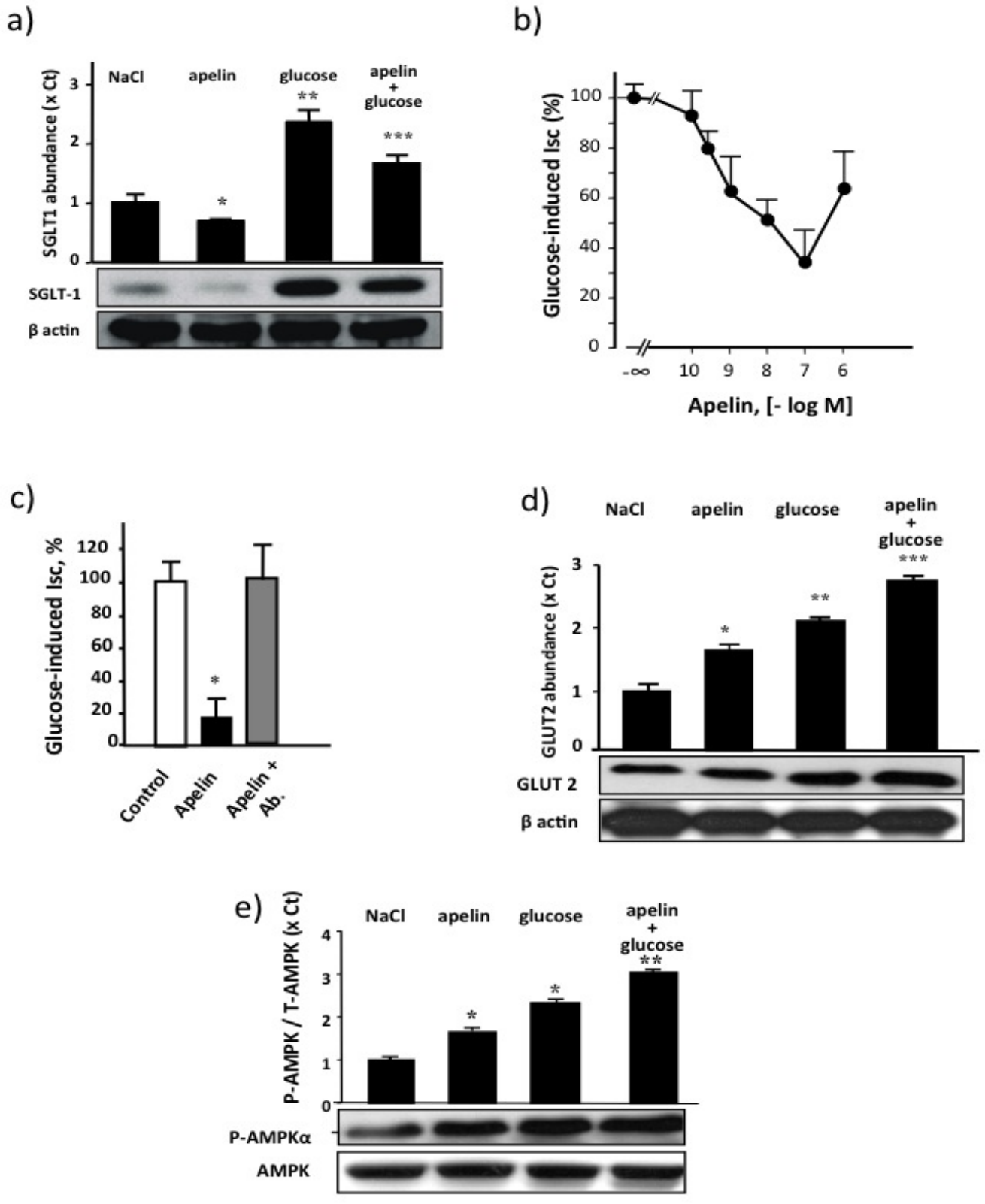

a)
α-APJ
ToPro III
Merge
α-IgG
b)
C1 C2 C3 A1 A2 A3
APJ
- 47 KDa
β-actin
- 42 KDa
Total APJ receptor abundance
2.0
1.5
1.0
0.5
0
n.s
PBS
Apelin
c)
C1 C2 C3 A1 A2 A3
APJ
- 47 KDa
β-actin
- 42 KDa
BBM APJ receptor abundance
2.0
1.5
1.0
0.5
0
*
PBS
Apelin

Fig.4


a)
NaCl
apelin
glucose
apelin + glucose
SGLT1 abundance (x Ct)
SGLT-1
β actin
b)
Glucose-induced Isc (%)
Apelin, [- log M]
c)
Glucose-induced Isc, %
Control
Apelin
Apelin + Ab.
d)
GLUT2 abundance (x Ct)
GLUT 2
β actin
e)
P-AMPK / T-AMPK (x Ct)
P-AMPKα
AMPK

1

Fig. 5

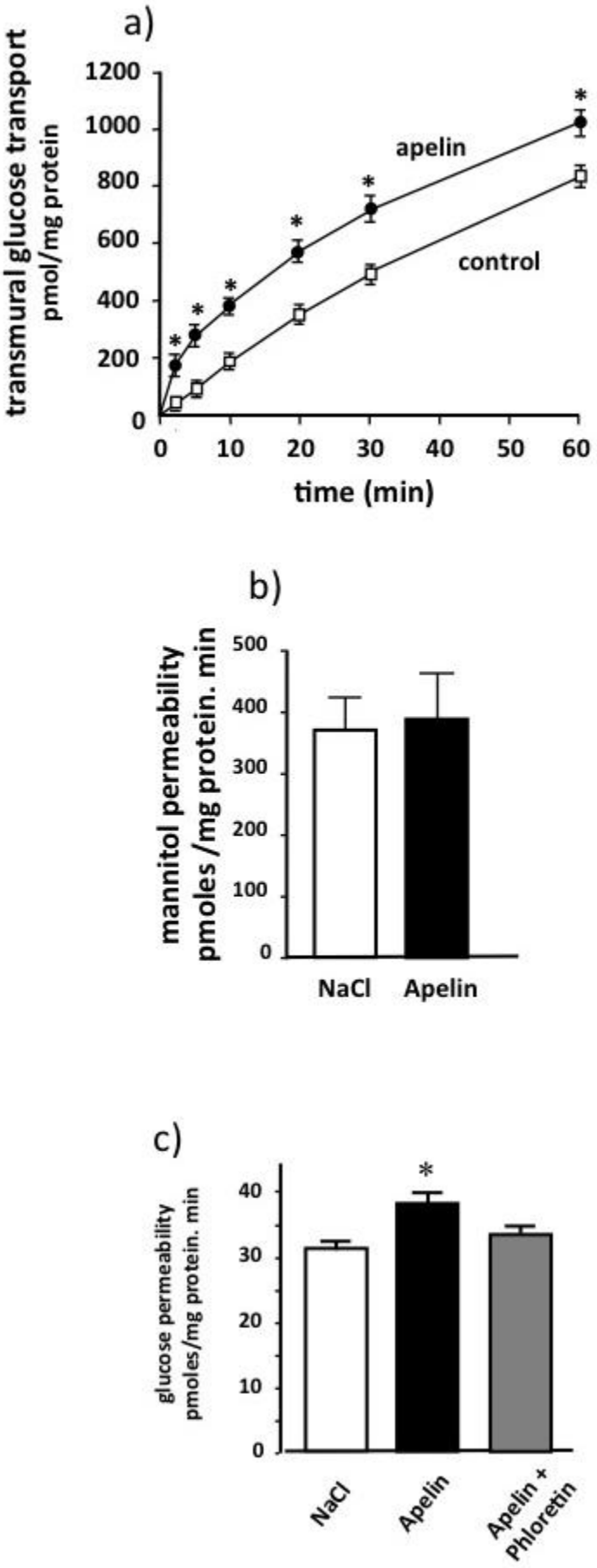
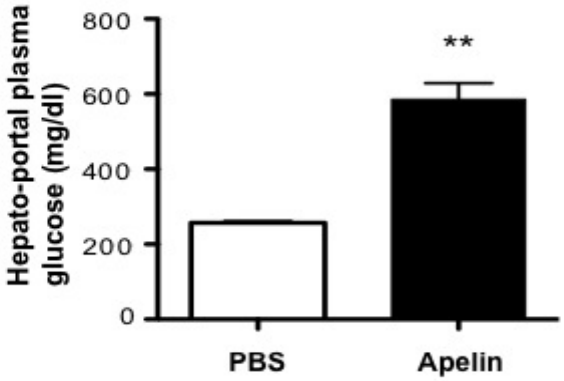
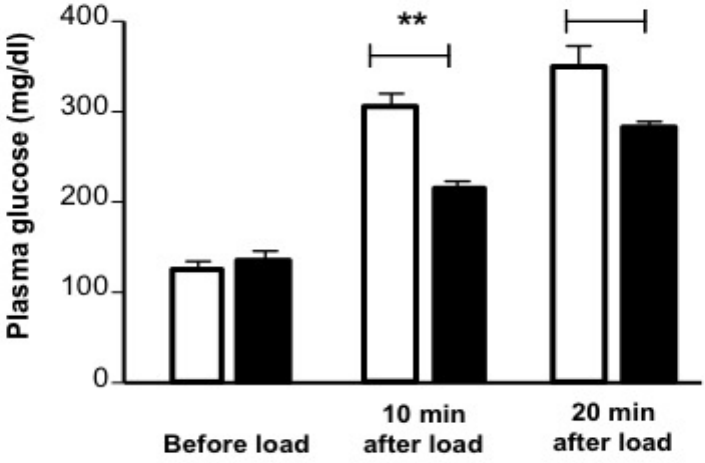

a)
transmural glucose transport
pmol/mg protein
1200
1000
800
600
400
200
0
apelin
control
*
0
10
20
30
40
50
60
time (min)
b)
mannitol permeability
pmoles /mg protein. min
500
400
300
200
100
0
NaCl
Apelin
c)
glucose permeability
pmoles/mg protein. min
40
30
20
10
0
*
NaCl
Apelin
Apelin +
Phloretin

Fig. 6

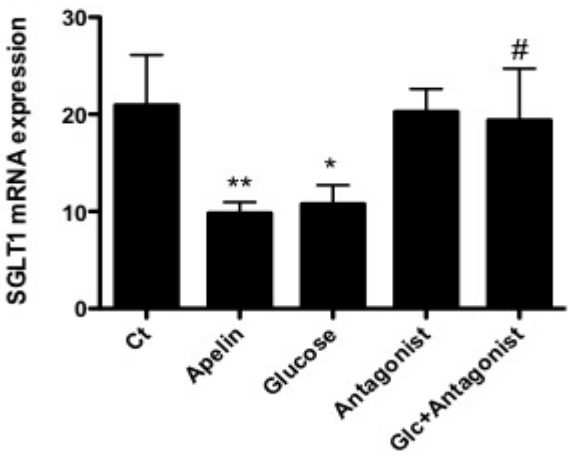
a)

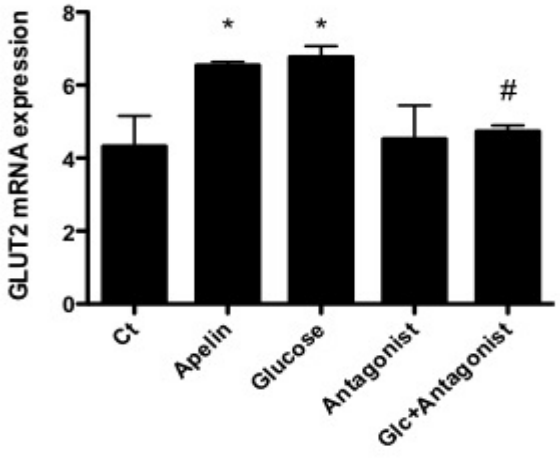

Hepato-portal plasma glucose (mg/dl)
800
600
400
200
0
**
PBS
Apelin

b)


Plasma glucose (mg/dl)
400
300
200
100
0
**
**
Before load
10 min after load
20 min after load

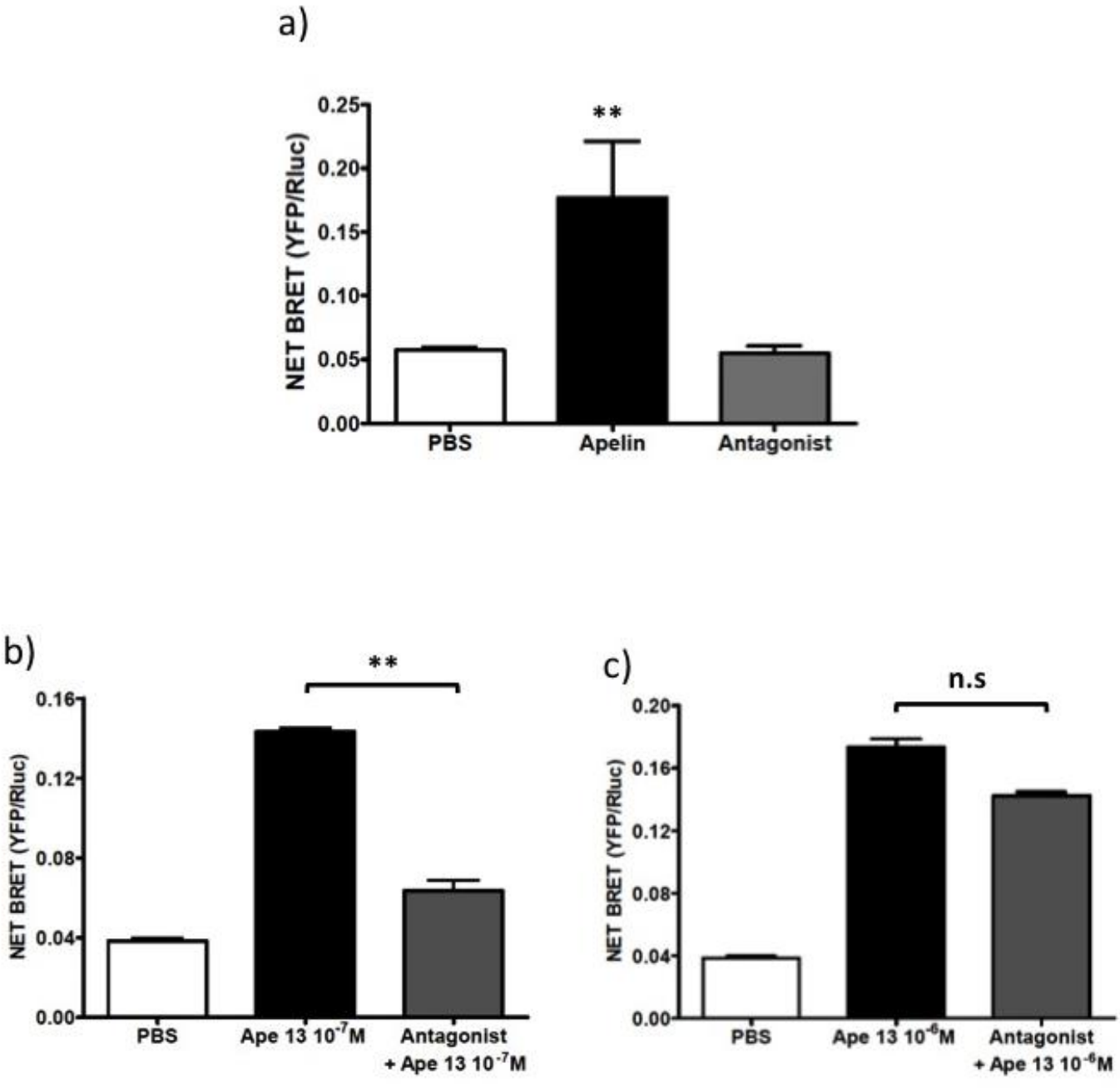
c)


SGLT1 mRNA expression
30
20
10
0
**
*
#
Ct
Apelin
Glucose
Antagonist
Glc+Antagonist


GLUT2 mRNA expression
8
6
4
2
0
*
*
#
Ct
Apelin
Glucose
Antagonist
Glc+Antagonist

1

Fig. 7


a)
NET BRET (YFP/Rluc)
0.25
0.20
0.15
0.10
0.05
0.00
**
PBS
Apelin
Antagonist
b)
NET BRET (YFP/Rluc)
0.16
0.12
0.08
0.04
0.00
**
PBS
Ape 13 10-7M
Antagonist + Ape 13 10-7M
c)
NET BRET (YFP/Rluc)
0.20
0.16
0.12
0.08
0.04
0.00
n.s
PBS
Ape 13 10-6M
Antagonist + Ape 13 10-6M

1

1