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# The apj receptor is expressed in pancreatic islets and its ligand, apelin, inhibits insulin secretion in mice

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**Abstract**

Apelin is the endogenous ligand of the G-protein coupled apj receptor. Apelin is expressed in the brain, the hypothalamus and the stomach and was recently shown also to be an adipokine secreted from the adipocytes. Although apelin has been suggested to be involved in the regulation of food intake, it is not known whether the peptide affects islet function and glucose homeostasis. We show here that the apj receptor is expressed in pancreatic islets and that intravenous administration of full-length apelin-36 (2 nmol/kg) inhibits the rapid insulin response to intravenous glucose (1g/kg) by 35% in C57BL/6J mice. Thus, the acute (1-5 min) insulin response to intravenous glucose was 682±23 pmol/l after glucose alone (n=17) and 445±58 pmol/l after glucose plus apelin-36 (n=18; P=0.017). This was associated with impaired glucose elimination (the 5-20 min glucose elimination was 2.9±0.1%/min after glucose alone versus 2.3±0.2%/min after glucose plus apelin-36, P=0.008). Apelin (2nmol/kg) also inhibited the insulin response to intravenous glucose in obese insulin resistant high-fat fed C57BL/6J mice (P=0.041). After 60 min incubation of isolated islets from normal mice, insulin secretion in the presence of 16.7 mmol/l glucose was inhibited by apelin-36 at 1 µmol/l, whereas apelin-36 did not significantly affect insulin secretion at 2.8 or 8.3 mmol/l glucose or after stimulation of insulin secretion by KCl. Islet glucose oxidation at 16.7 mmol/l was not affected by apelin-36. We conclude that the apj receptor is expressed in pancreatic islets and that apelin-36 inhibits glucose-stimulated insulin secretion both in vivo and in vitro. This may suggest that the islet beta-cells are targets for apelin-36.

**Key words: Apelin, apj receptor, insulin secretion, islets, mice**

## 1. Introduction

The apj receptor was cloned in 1993 and found to exhibit significant homology with the angiotensin II receptor type 1, although angiotensin is not a ligand of the apj receptor (1). The apj receptor was originally shown to be expressed in several regions in the CNS (1,2). It was subsequently demonstrated to be ubiquitously expressed with high expression levels in the lung, heart, medial layer of vessels and endothelial cells (3,4). The receptor is a G-protein coupled receptor signaling inhibition of adenylyl cyclase activity eliciting reduced cAMP formation (1,5) although also raise of intracellular calcium and activation of extracellularly regulated kinases (ERK) have been reported (6,7). The endogenous ligand for the apj receptor is apelin, which was initially demonstrated in stomach extracts in 1998 (8). Later studies have shown that apelin mRNA is expressed in several tissues, as for example in several regions in the CNS, in the heart, lung, placenta, mammary gland and gastrointestinal tract (3,5,9-11). Secretion of apelin has been demonstrated from the adipocytes (12) and in the colostrums (5). Apelin is a 36 amino acid peptide, which is processed from a 77 amino acid proapelin (8). However, multiple apelin forms exist consisting of 13, 16 and 17 amino acids with the C-terminal end identical in all forms (13); in fact, the C-terminal end is highly conserved across species (5).

The physiological role of apelin/apj receptor activation is not known. Since the receptor and the ligand are co-localized in many tissues, like the heart, lung and various regions in the CNS (3), a paracrine pattern of regulation by apelin is possible. The peptide may also exhibit neural actions due to its high abundancy in the hypothalamus (14). Functionally, actions of apelin start to emerge as evident in experimental studies (for a review see 3). For example, apelin has been shown to exert cardiovascular effects including positive inotropic action and to reduce blood pressure, as demonstrated in rodents (3,15,16). Apelin also increases drinking behavior (14) and reduces food intake (17) in model experiments in rodents. Furthermore,

mice with null mutation of the apj receptor gene exhibit increased vasopressor response to angiotensin II as suggestive of counter-regulation of the apj and angiotensin II receptors (18). It was recently reported that apelin is expressed in adipocytes (12), which suggests that apelin may function as an adipokine. The adipocyte expression of apelin was also found to be increased in rodent models of obesity and reduced in streptozotocin-induced diabetes as well as after fasting, and augmented by insulin, suggesting that insulin is an important regulator of apelin expression (12). Also circulating apelin levels were found to be increased in obesity (12). This would suggest that apelin may be an adipokine involved in the perturbation of metabolism associated to obesity.

This study examined whether apelin may be involved in the regulation of glucose homeostasis, which would be a possibility due to its expression in the stomach, in adipocytes and in the hypothalamus, and because the pancreatic islets are under control of factors derived from the gastrointestinal tract, adipocytes and the autonomic nerves (19-22). We thus examined whether the apj receptor is expressed in pancreatic islets and whether exogenous administration of full-length apelin (apelin-36) affects the insulin response to an intravenous glucose challenge in mice and insulin secretion from isolated mouse islets.

## 2. Materials and methods

### *2.1 Animals.*

Female C57BL/6J mice, were purchased from Taconic (Skensved, Denmark). The animals were maintained in a temperature-controlled room (22ºC) on a 12h light-dark cycle. Mice were fed a standard pellet diet (Lactamin, Stockholm, Sweden) and tap water ad libitum. The experiments were performed at the age of 3-4 months (body weight 28.3±0.9 g, range 20.6-40.1 g), except in one series of experiments, when C57BL/6J mice were fed a high-fat diet for 8 months, whereafter an intravenous glucose tolerance test was undertaken (body weight 43.6±1.6g, range 29.7-56.5 g). On a caloric basis, the high-fat diet consisted of 58% fat from lard, 25% carbohydrate and 16% protein (total 23.4 kJ/g; Research Diets, New Brunswick, NJ), whereas the control diet contained 11.5% fat, 73% carbohydrate and 16.4% protein (total 17.2 kJ/g). The study was approved by the Local Ethic Animal Committee, Lund, Sweden.

### *2.2 Islet apj recepto  mRNA expression*

Total RNA was extracted from isolated islets from 5 mice using RNeasy Mini Kit (Qiagen, Hilden, Germany) and control RNA was extracted from mouse spleen. The RNA was DNase treated using Deoxyribonuclease I (according to manufacturer's protocol, Invitrogen, Carlsbad, CA). First strand cDNA synthesis was then transcribed with Superscript II reverse transcriptase (RT, according to manufacturer's protocol, Invitrogen, Carlsbad, CA) and random hexamer primers (Takara, Shiga, Japan). As controls for contaminating DNA identical transcription reactions were set up without RT. Apj receptor primers (forward primer 5'-CTC AGC AGC TAC CTC ATC TTT GT, reverse primer 5'-TGA AGT GGC CAC CAT AGA GTA GT, Invitrogen, Paisley, Scotland) were design to amplify a 264 bp transcript corresponding to nucleotides 515-778 in the apj receptor mRNA sequence (GenBank Accession no. AK045406). A polymerase chain reaction of 35 cycles was performed on the

transcribed cDNA using Platinum Taq Polymerase according to the manufacturer with an annealing temperature of 61°C (Invitrogen, Carlsbad, CA). PCR-fragments were gel purified using GFX PCR DNA and gel band purification kit (Amersham Biosciences Ltd, Buckinghamshire, Egland) prior to sequencing with primer elongation at Cybergene AB, Huddinge, Sweden.

*2.3 In vivo experiments*

Mice were anesthetized after a 3 hr fasting period during the late morning hours with an intraperitoneal injection of midazolam (Dormicum®, Hoffman-La-Roche, Basel, Switzerland, 0.14 mg/mouse) and a combination of fluanison (0.9 mg/mouse) and fentanyl (0.02 mg/mouse; Hypnorm®, Janssen, Beerse, Belgium). After 30 min, a blood sample (75 µl) was taken from the retrobulbar, intraorbital, capillary plexus in a 100 µl pipette which had been pre-rinsed in heparin solution (100 U/ml in 0.9% NaCl; Lövens, Ballerud, Denmark). Thereafter, either saline (n=18) or D-glucose alone (1 g/kg, n=17, Sigma Chemicals, St Louis, MO), or D-glucose together with synthetic apelin-36 (2 nmol/kg, n=18, Phoenix Pharmaceuticals, Belmont, CA), or apelin-36 alone were injected in a tail vein. The volume load of the injections was 10 µL/g body weight. Blood samples (75 µL each) were taken at 1, 5, 10, 20, 30 and 50 min. Plasma was immediately separated and stored at –20°C until analyses.

*2.4 Islet isolation, insulin secretion and glucose oxidation*

Islets were isolated from the pancreas by collagenase digestion and handpicked under microscope. Batches of islets were preincubated in HEPES balanced salt solution (HBSS) containing 125 mM NaCl, 5.9 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM HEPES (pH 7.4), 2.8 mM glucose and 0.1% fatty acid free bovine albumin (Boehringer Mannheim, GmbH, Germany) for 60 min. Thereafter, islets in groups of three were incubated in 200 µl of the HBSS with varying concentrations of glucose ± apelin-36 (10 nmol/l and 1 µmol/l) or with

glucose (16.7 mmol/l) together with KCl (35 or 50 mmol/l) ± apelin-36 (1 μmol/l), and were incubated for 60 min at 37°C. After the incubation, aliquots of 25 μl in duplicates were collected and stored at -20°C until analysis of insulin. For measurement of glucose oxidation, batches of 30 islets were incubated with 0.1 μCi or 0.7 μCi [$^{14}$C]-glucose (NEN, Boston, MA, specific activity 310 mCi/mmol) and 2.8 mM or 16.7 mM glucose, respectively, with and without 1 μmol/l apelin-36. The reaction was terminated after 2 h and the amount of released $^{14}CO_2$, trapped with benzetonium hydroxide, was determined by scintillation counting.

*2.5 Assays*

Insulin concentration was determined by a double-antibody radioimmunoassay using guinea pig anti rat insulin antibodies, $^{125}$I-labelled human insulin and, as standard, rat insulin (Linco Res., St. Charles, MO, USA). Glucose was measured by the glucose oxidase technique.

*2.6 Data handling and statistics*

Means±SEM are shown. The rate of glucose disappearance after bolus glucose administration was evaluated by the glucose tolerance index $K_G$ (%/min), calculated as the slope of the regression *vs.* time of the logarithmically transformed glucose concentration values from 5 to 20 min. The acute insulin response to intravenous glucose (AIR) was calculated as the suprabasal mean 1 and 5 min insulin levels. The suprabasal area under the 75 min insulin curve ($AUC_{insulin}$) was calculated by the trapezoid rule. Statistical comparisons between groups were performed with ANOVA with Bonferroni *post hoc* analysis.

## 3. Results

### *3.1 Islet apj receptor mRNA expression*

Apj receptor mRNA is expressed in pancreatic islets and the islet expression level appears to be slightly lower than the expression in the spleen (Fig. 1). No bands were obtained in the DNA controls and genomic DNA contamination was excluded. The amplified PCR-fragment was sequenced and found to be 100% identical to the reported apj receptor sequence (GenBank Accession no. AK045406).

### *3.2 Intravenous glucose*

The first series of experiments were undertaken in normal C57BL/6J mice aged 8 weeks fed a normal diet. Fig. 2 shows that following the intravenous administration of glucose (1 g/kg) in these mice, plasma insulin was rapidly increased to a peak level at 1 min. The rapid initial insulin response lasted for 5 min. Apelin-36 (2 nmol/kg) reduced the glucose-induced increase in insulin levels. Thus, the 1 min peak was 1369±103 pmol/l after glucose alone (n=17) but only and 932±91 pmol/l after glucose plus apelin-36 (n=18; P=0.003). The AIR was reduced by 35% by apelin-36, being 682±73 pmol/l after glucose alone versus 445±58 pmol/l after glucose plus apelin-36 (P=0.017). Concomitantly, glucose elimination was reduced by apelin-36, resulting in significantly higher glucose levels at 10 minutes (P=0.039) and 20 minutes (P=0.006) in the group given apelin-36 plus glucose versus the group given glucose alone. The glucose elimination rate between min 5 and 20, $K_G$, was 2.9±0.1%/min in the control group given glucose alone versus 2.3±0.2%/min after glucose plus apelin-36 (P=0.008). Fig. 2 also shows that intravenous administration of saline (n=18) slightly increases circulating glucose from 8.7±0.3 mmol/l to 9.5±0.2 mmol/l after 75 minutes (P=0.048), whereas plasma insulin was not significantly altered (141±9 pmol/l before saline administration versus 152±12 pmol/l after 75 minutes, P=0.38). Apelin-36 alone (2 nmol/kg; n=18) had no effect on these basal levels of glucose and insulin.

The second series of experiments was undertaken in mice given a high-fat diet for 8 months. Fig. 3 shows that the insulin response to the intravenous glucose was reduced by apelin-36 in the high-fat fed mice; the $AUC_{insulin}$ being 46.5±21.9 nmol/lx75 min after glucose alone (n=7) versus –25.6±22.2 nmol/lx75 min after glucose and apelin-36 (n=6; P=0.041).

*3.3 Insulin secretion and glucose oxidation in vitro*

Fig. 4A shows that insulin secretion from islets isolated from the normal mice in the presence of 16.7 mmol/l glucose was inhibited by apelin-36 at 1 μmol/l, whereas apelin-36 had no significant effect at 2.8 or 8.3 mmol/l glucose. In contrast, when islets were incubated in high KCl (35 or 50 mmol/l) in the presence of glucose at 16.7 mmol/l, apelin-36 (1 μmol/l) did not reduce insulin secretion (Fig. 4B). To examine whether the inhibitory action of apelin-36 on glucose-stimulated insulin secretion may be associated with reduced glucose metabolism, we also examined the oxidation of glucose in the islets. As expected, islets incubated at 16.7 mmol/l glucose had markedly higher glucose oxidation (3.19±0.27 pmol/islet/h, n=8) than islets incubated at 2.8 mmol/l glucose (0.65±0.15 pmol/islet/h, n=8, P<0.001). However, apelin-36 (1 μmol/l) did not affect glucose oxidation (being 0.59±0.08 pmol/islet/h at 2.8 mmol/l glucose (n=8) and 2.82±0.24 pmol/islet/h at 16.7 mmol/l (n=8), both not significant versus control).

## 4. Discussion

Insulin secretion is regulated by a number of factors to ensure a normal insulin levels for maintaining a normal glucose homeostasis. Factors from the gastrointestinal tract are of marked importance, both as stimulators of insulin secretion (for example glucagon-like peptide-1 and other incretins; 19) and as inhibitors of insulin secretion (for example ghrelin, 23). Also neural factors localized to the hypothalamus are of importance, as the autonomic nervous system exerts both stimulatory (parasympathetic effects) and inhibitory (sympathetic) effects on insulin secretion (22). Finally, adipocyte derived adipokines, such as adiponectin and leptin, also affect islet function (20,21). Although the function of apelin-36 still remains to be established, the peptide is of interest in this respect due to its localization to the stomach (8,10), adipocytes (12) and the hypothalamus (14). Its proposed action to be involved in the regulation of food intake (17) is also of interest in this context, because many bioactive peptides have the dual action to regulate food intake and islet function.

Here we show that apelin-36 inhibits glucose-stimulated insulin secretion both in vivo and in vitro in mice. In vivo, the peptide reduced the increase in circulating insulin after an intravenous glucose challenge. The effect was potent eliciting a 35% inhibition of the response in the normal mice, which in turn was associated with impaired glucose elimination. Apelin also inhibited the insulin response to intravenous glucose in high-fat fed mice. These mice are obese and insulin resistant and the high-fat fed mouse model has been shown to be a good model for studies of islet perturbations due to insulin resistance (24). The inhibition of glucose-stimulated insulin secretion by apelin would fit activation of the sympathetic nervous system, since such an activation is associated with impaired insulin secretion (22). The high abundancy of apj receptor mRNA in hypothalamus (9,14) with high expression in the paraventricular and supraoptic nuclei (9) would support a role of apelin in the central regulation of hormone release. The previous finding that apelin reduces blood pressure in

rodents would also support such an indirect action, albeit at very high dose levels (>10 nmol/kg; 25), since reduced blood pressure adaptively augments activation of the sympathetic nervous system. However, we also show the expression of apj receptor mRNA in isolated islets, which would suggest a direct effect of apelin on beta-cells. This is supported by our results that apelin-36 inhibits glucose-stimulated insulin secretion from isolated mouse islets. The mechanism of action underlying the inhibitory action of apelin on glucose-stimulated insulin secretion is not known. We found that this action of apelin was seen without any effect on islet glucose metabolism, as evident from determinations of glucose oxidation, and furthermore, that apelin did not inhibit insulin secretion induced by depolarization by high potassium. Therefore, more studies are required to elucidate the mechanism.

In our study it was not possible to distinguish in which islet cell type apj receptor mRNA is expressed. Low apj mRNA expression has previously been shown in human pancreas but it was not detected in rat pancreas (11). A possible species difference could account for this discrepancy, or it could be due to a dilution effect when the expression was studied in whole pancreas, since it is possible that the apj receptor only is expressed in islet cells. Our expression study is solely qualitative and we have not determined the quantitative expression of apj receptor mRNA in islets or in other tissues.

Our results therefore suggest that apelin may be involved in the regulation of islet function, although the role of apelin remains to be established. It is in this context of particular interest that apelin recently was identified in adipocytes and that the expression of the peptide is increased in obesity (12). Whether apelin and insulin thereby are mutually involved in a feed back autoregulation needs to be studied. It may also be emphasized that apelin shows similarities to the adipokine leptin, because also the expression in adipocytes of this hormone is increased in obesity (20) and leptin, as now demonstrated by apelin, has been shown to inhibit insulin secretion (20,26). Of interest is also that beta-cells have been shown to express

the angiotensin receptor 1, which is similar to the apj receptor, and the effect of angiotensin in isolated islets was a reduced insulin secretion response after glucose stimulation (27). The functional relevance of this remains, however, to be studied.

In conclusion, our results show that the apj receptor is expressed in pancreatic islets and that apelin-36 inhibits insulin both in vivo and in vitro in mice. This suggests that the novel adipokine apelin may be involved in the regulation of islet function. Further studies are now warranted to establish the role of this insulinostatic action of apelin-36 and its relation to obesity, diabetes and food intake.

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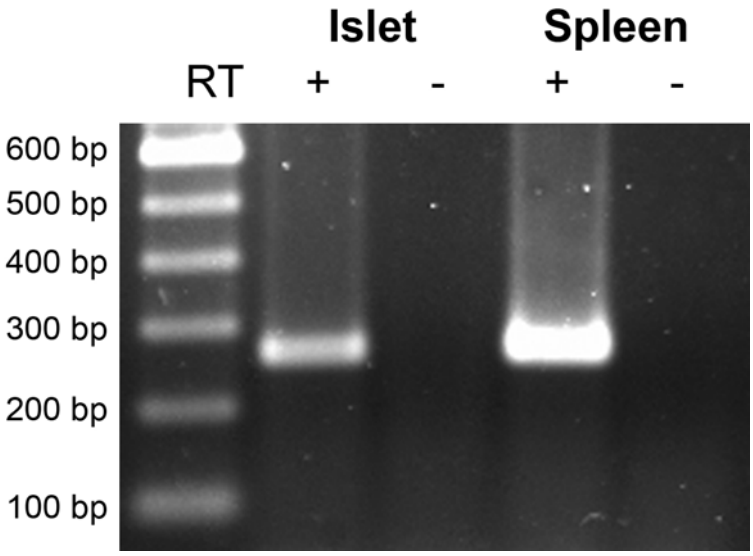
**Legends to the Figures**

**Fig. 1** Expression of apj receptor in islets and spleen visualized on a 2% agarose gel by CYBR green® staining. PCR-fragments were amplified from cDNA, which was transcribed from DNase treated total RNA. As controls contaminating DNA, cDNA samples without reverse transcriptase (-RT) were used.

**Fig. 2** Plasma levels of glucose (upper panel) and insulin (lower panel) before and after intravenous administration of saline or glucose (1 g/kg) alone or together with apelin-36 (2 nmol/kg) in female C57BL/6J mice. There were 17-18 animals in each group. Means±SEM are shown. Asterisks indicate probability level of random difference between the groups (*P<0.05; **P<0.01).

**Fig. 3** Plasma levels of glucose (upper panel) and insulin (lower panel) before and after intravenous administration of saline or glucose (1 g/kg, n=7) alone or together with apelin-36 (2 nmol/kg, n=6) in female C57BL/6J mice fed a high-fat diet for 8 months. Means±SEM are shown.

**Fig. 4** Medium insulin concentrations after 60 min incubation of islets isolated from female C57BL/6J mice. Upper panel shows results after incubation of islets in the presence of 2.8, 8.3 or 16.7 mmol/l glucose alone or together with apelin-36 at 10 nmol/l or 1 μmol/l. There were 8-16 incubations with three islets in each incubation for each experimental group. Lower panel shows results after incubation of islets in the presence of 2.8 or 16.7 mmol/l glucose alone or glucose at 16.7 mmol/l together with KCl at 35 or 50 mmol/l with or without apelin-36 at 1 μmol/l. There were 16 incubations with three islets in each incubation for each experimental group. Means±SEM are shown.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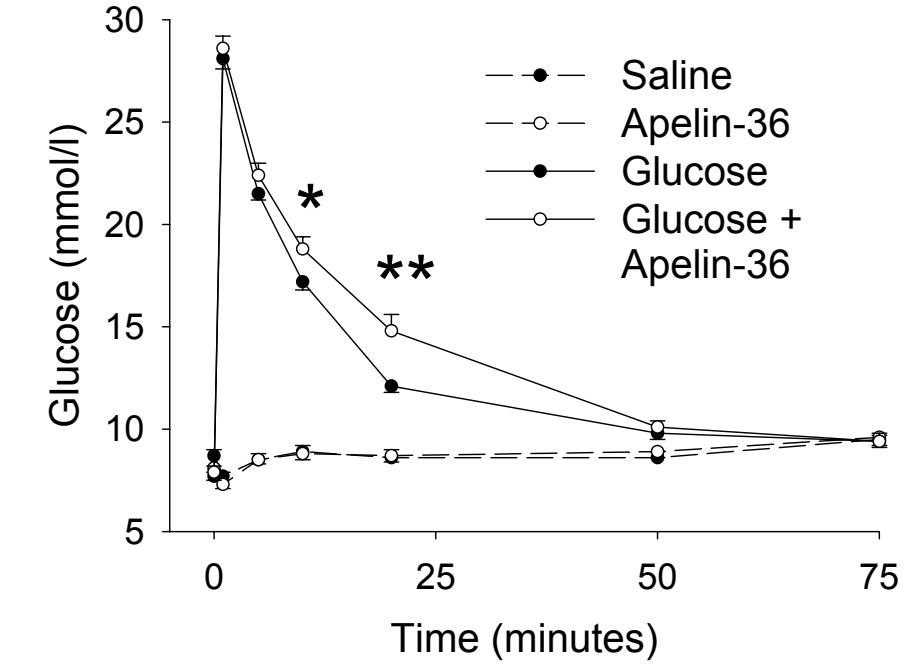
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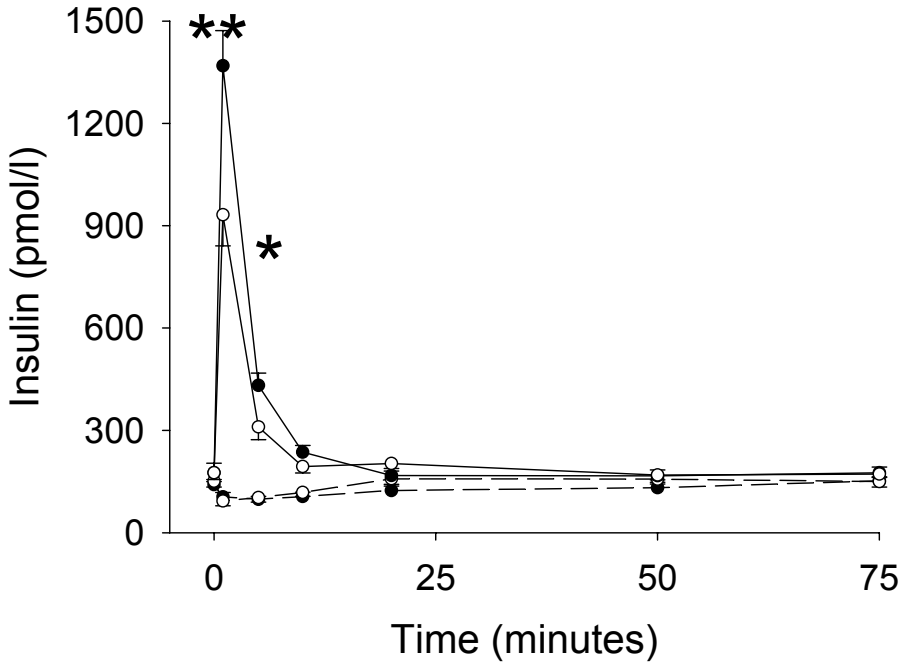
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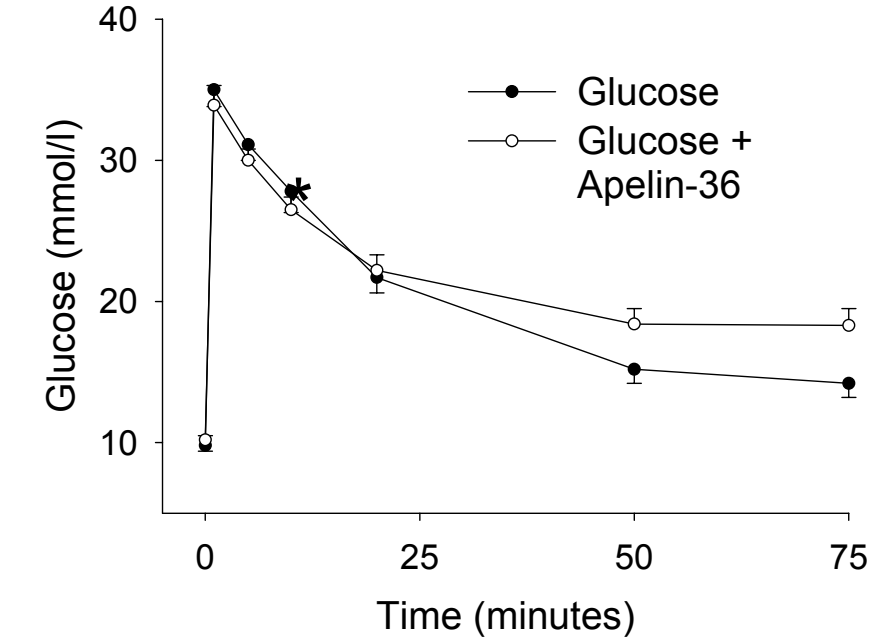
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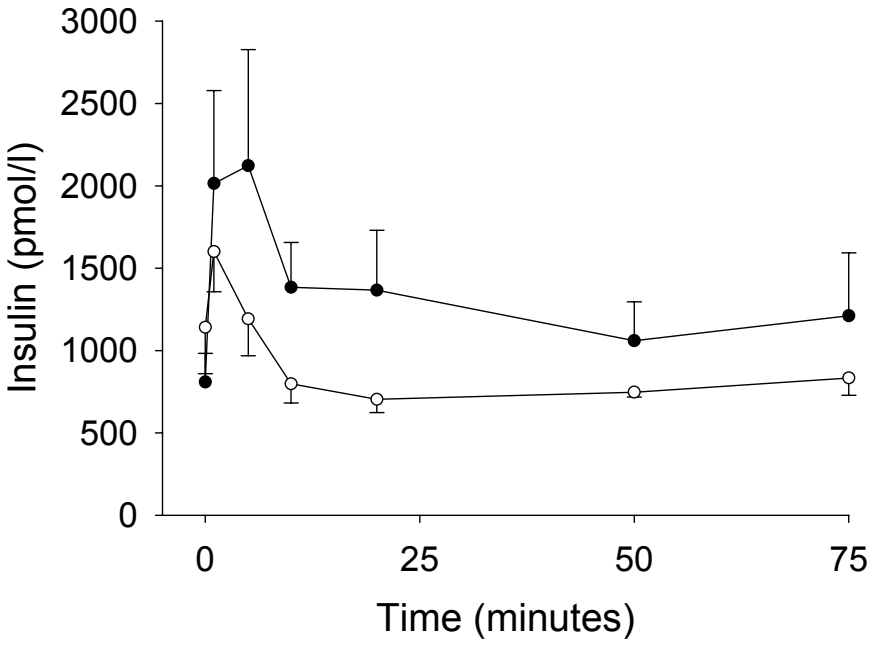
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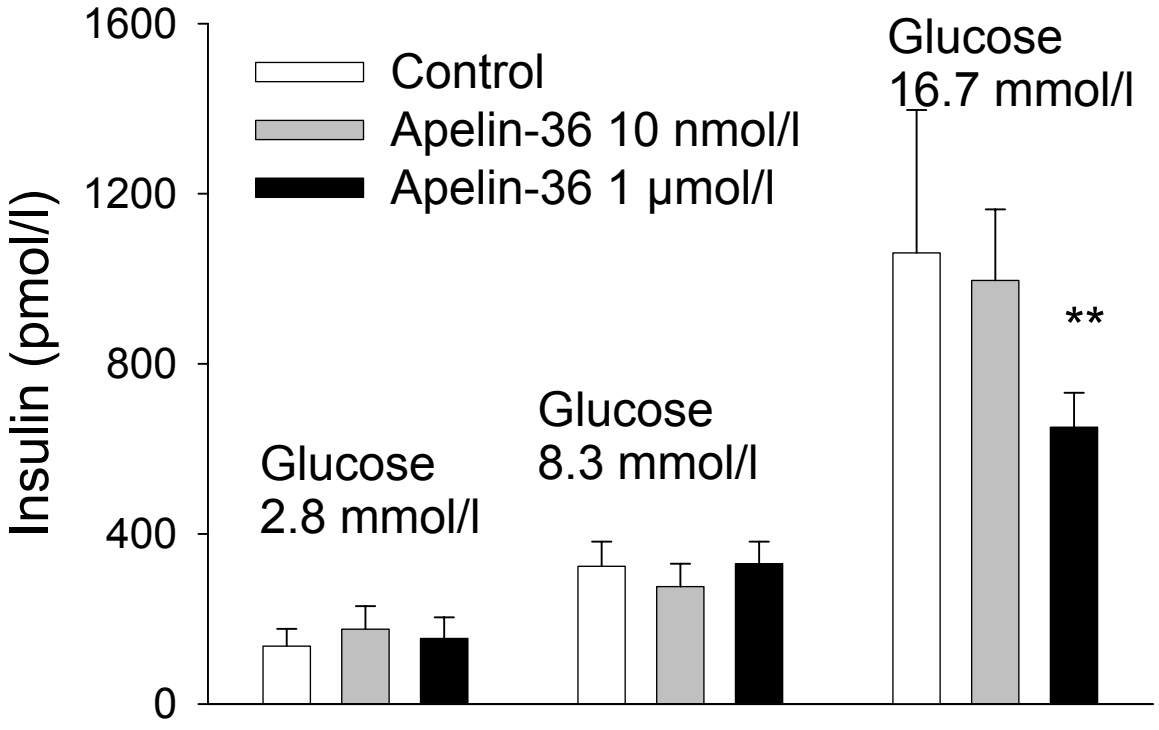
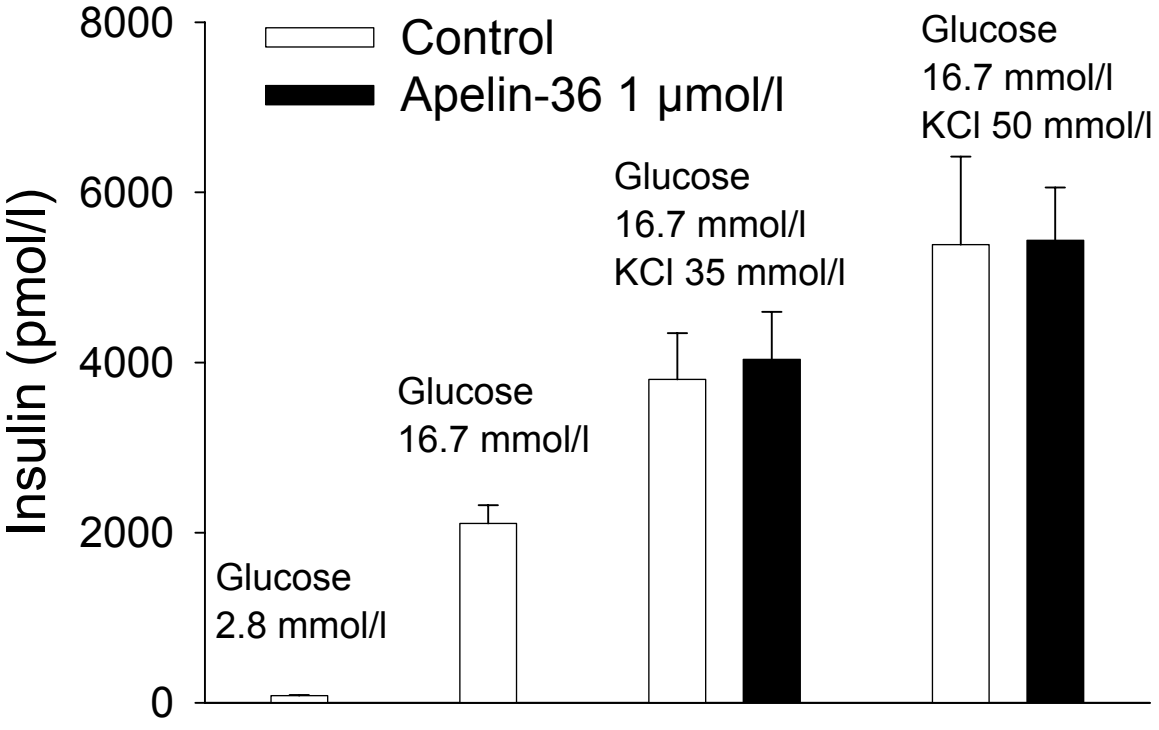
75
Time (minutes)


1600
1200
800
400
0
Insulin (pmol/l)
Control
Apelin-36 10 nmol/l
Apelin-36 1 µmol/l
Glucose
16.7 mmol/l
**
Glucose
8.3 mmol/l
Glucose
2.8 mmol/l


8000
6000
4000
2000
0
Insulin (pmol/l)
Control
Apelin-36 1 µmol/l
Glucose
16.7 mmol/l
KCl 50 mmol/l
Glucose
16.7 mmol/l
KCl 35 mmol/l
Glucose
16.7 mmol/l
Glucose
2.8 mmol/l