Amylin reduces plasma glucagon concentration in cats

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Abstract

Pancreatic amylin plays an important role in the control of nutrient fluxes and is an established therapy in human diabetes as it reduces post-prandial glucagon secretion and slows gastric emptying. Given the similar pathophysiology of human type-2 and feline diabetes mellitus, we investigated whether amylin reduces plasma glucagon levels in cats. Healthy cats were tested using an intravenous arginine stimulation test (IVAST), a meal response test with the test meal comprising 50% of average daily food intake, and an IV glucose tolerance test (IVGTT). Non-amyloidogenic rat amylin injected 5min before the respective stimulus significantly reduced plasma glucagon levels under all test situations. In the IVAST and IVGTT, cats treated with amylin also had lower plasma insulin concentrations. It was concluded that amylin does reduce plasma glucagon levels in cats, a feature that has treatment potential in diabetic animals as co-administration of amylin would reduce the insulin requirement to control glycaemia.
Amylin reduces plasma glucagon concentration in cats

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Abstract

Pancreatic amylin plays an important role in the control of nutrient fluxes and is an established therapy in human diabetes as it reduces post-prandial glucagon secretion and slows gastric emptying. Given the similar pathophysiology of human type-2 and feline diabetes mellitus, we investigated if amylin reduces plasma glucagon levels in cats. Healthy cats were tested using an intravenous (IV) arginine stimulation test (IVAST), a meal response test (MRT) with the test meal comprising 50% of average daily food intake, and an IV glucose tolerance test (IVGTT). Non-amyloidogenic rat amylin injected 5 min before the respective stimulus significantly reduced plasma glucagon levels under all test situations. In the IVAST and IVGTT, cats treated with amylin also had lower plasma insulin concentrations. We conclude that amylin does reduce plasma glucagon levels in cats, a feature that has treatment potential in diabetic animals as co-administration of amylin would reduce the insulin requirement to control glycaemia.

Keywords: Amylin; Glucagon; Cat; Diabetes mellitus
Introduction

Diabetes mellitus (DM) is a common endocrinopathy in cats. Its prevalence has risen over the last 30 years possibly reflecting the higher prevalence of obesity in this species. Cats are an interesting model of DM in that they spontaneously develop a form of the disease similar to human type-2 DM (T2DM) (Lutz and Rand, 1995; Cefalu, 2006; Henson and O’Brien, 2006). Diabetic cats are often middle aged and, similar to human T2DM, obesity with concurrent insulin resistance is a major risk factor, and impaired beta-cell function is present. The most striking histological similarity between human T2DM and the disease in cats is the amylin-derived islet amyloidosis that is found in > 80% of diabetic cats (Lutz and Rand, 1995). Primates and felines develop islet amyloidosis due to the amyloidogenic amino acid sequence of amylin while rodents do not spontaneously develop T2DM-like disease as their amylin is non-amyloidogenic (Westermark et al., 1990; Johnson et al., 1992).

Amylin is a normal secretory product of pancreatic beta-cells, co-synthesised and co-secreted with insulin in response to appropriate stimuli (Lutz and Rand, 1996; Young, 2005a) so that plasma insulin and amylin levels usually parallel each other. Early-stage or mild feline DM may be characterised by compensatory hyperinsulinaemia and absolute or relative hyperamylnaemia (O’Brien et al., 1991; Lutz and Rand, 1996). In such circumstances hyperamylnaemia may favour the deposition of amylin as pancreatic amyloid. Frequently however, clinical cases of feline DM are presented to veterinarians in more severe or late-stage disease by which time progressive beta-cell failure has resulted in overt hypoinsulinaemia and hypoamylnaemia (Johnson et al., 1989; Ludvik et al., 1991).
Amylin plays an important role in controlling nutrient metabolism through inhibiting pancreatic glucagon release and food intake (Lutz, 2005), and through slowing gastric emptying (Edelman and Weyer, 2002). In humans and in rodent animal models, lack of amylin in DM thus results in over-secretion of glucagon, over-eating and accelerated gastric emptying. Amylin reduces the excessive post-prandial hyperglucagonaemia observed in DM (Fineman et al., 2002) and normalises gastric emptying in human diabetics treated with pramlintide, a non-amyloidogenic amylin analogue (Thompson et al., 1998; Weyer et al., 2001; Fineman et al., 2002; Ratner et al., 2002; Vella et al., 2002; Hollander et al., 2003, 2004; Maggs et al., 2004).

Hyperglucagonaemia is also a feature of diabetic cats (Tschuor et al., 2006), but it remains unknown if this is due to the lack of amylin. Given the similar pathophysiology of human T2DM and feline diabetes, and given that human diabetics are successfully treated with a combination of amylin and insulin, it seems pertinent to study the effect of amylin on glucagon secretion in cats. In this study we investigate the effect of amylin injection on plasma glucagon levels in healthy cats under three test conditions: an intravenous (IV) arginine stimulation test (IVAST); a meal response test (MRT); and an IV glucose tolerance test (IVGTT). Plasma glucagon levels increased in response to arginine injection and to feeding but were reduced following IV glucose administration. In our study we used rat amylin because of its non-amyloidogenic properties (Westermark et al., 1990; Johnson et al., 1992) and assessed if it reduced plasma glucagon concentrations under these test conditions in healthy cats. The doses of amylin used (5 or 10 µg/kg) were based on effective doses previously used in rats and humans (Young et al., 1996; Hollander et al., 2003; Lutz, 2005).
Materials and Methods

The procedures were carried out on 12 male domestic short-hair cats (Harlan Sprague Dawley) that had been castrated between 5-10 months of age and were between 16-24 months old at the time of study. Their average bodyweight was 4.9 ± 0.4 kg. All experimental procedures were approved by The Cantonal Veterinary Office, Zurich, Switzerland (licence number 213/2003 ZH).

During the study, cats were group-housed except when under test and were given a four week adaptation period prior to the start of the experiment. The animals were kept in artificial light with the light on from 07:00-19:00 and were offered commercial canned food (Purina Veterinary Diet, Diabetes Management, Nestlé Purina) twice daily. Food consumption was such that the cats maintained a stable bodyweight which was recorded weekly. Water was available ad libitum.

Implantation of jugular catheter

A 10 cm long jugular catheter (4 French polyurethane catheter, Global Veterinary Products) was inserted using the Seldinger technique (Seldinger, 1953) and sutured to the skin on the day prior to testing. Cats were sedated with ketamine (Ketaminol, Veterinaria) at a dose of 5-7 mg/kg intramuscular (IM) and midazolam (Dormicum, Roche) at 0.2 mg/kg (IM), followed by IV propofol (Fresenius Kabi) at 6-7 mg/kg to anaesthetise.

Pharmacokinetic of rat amylin in cats

We determined the pharmacokinetics of rat amylin in four cats in a pilot experiment. Rat amylin (Amylin Pharmaceuticals Inc.) was used as, in contrast to cat amylin, it is non-
amyloidogenic. The dose of amylin used (5 µg/kg dissolved in 0.1 mL/kg saline) was based on that effective in rats and humans (Young et al., 1996; Hollander et al., 2003; Lutz, 2005) and was administered subcutaneously (SC). Blood samples were taken into EDTA tubes 5, 10, 15, 20, 25 and 30 min after injection and centrifuged immediately. Aprotinine (Trasylol, Bayer) was added to the EDTA-plasma mixture at 500 kIU/mL. Plasma samples were frozen at –80 °C until further analysis.

**Intravenous arginine stimulation test**

The IVAST was used to investigate the effect of amylin on arginine induced glucagon secretion. Six cats per group were fasted overnight for 12 h before testing. After taking a 2 mL basal blood sample, amylin (5 or 10 µg/kg) or saline solution (control treatment, 0.1 mL/kg) were injected SC. Five min following amylin or saline administration (t = 0), L-arginine (0.2 g/kg, L-arginine hydrochloride 21%, B. Braun Melsungen) was injected via the catheter. Blood samples were taken 2, 4, 7, 9, 15, 25 and 30 min after L-arginine injection. These sampling points were chosen based on previous studies (Tschuor et al., 2006). Volume replacement was achieved by injecting 0.9% saline solution. Blood was taken into fluoride blood tubes (to measure glucose concentrations), and into EDTA tubes (to measure insulin and glucagons concentrations) and was centrifuged immediately. EDTA-plasma was treated as described above.

**Meal response test**

Six cats per group were fasted for 24 h before the MRT which was carried out at the onset of the light period. After taking a 2 mL basal blood sample, amylin (5 µg/kg) or saline solution (control treatment, 0.1 mL/kg) were injected SC and 5 min later, the test meal comprising 50% of the average daily food intake was offered. All food was consumed within 10
min and at this time (t = 0), a second blood sample was obtained. Further samples were taken 15
and 30 min and 1, 2 and 5 h later, sampling time-points based on pilot studies. Volume
replacement was achieved by injecting 0.9% saline solution and blood samples were treated as
described above.

*Intravenous glucose tolerance test*

Six cats per group were fasted for 12h overnight before the IVGTT. After taking a 2
mL basal blood sample, amylin (5 or 10 µg/kg) or saline solution (control treatment, 0.1 mL/kg)
were injected SC and 5 min later (t = 0), D-glucose (0.5 g/kg, 50 % sterile dextrose; 1 mL/kg)
was injected slowly over 30 s via the catheter. Blood samples were taken 3, 7, 10, 15, 30, 60, 120
and 180 min after glucose injection, sampling time-points based on previous studies (Lutz and
Rand, 1996). Volume replacement was achieved by injecting 0.9% saline solution and blood
samples were treated as described above.

*Biochemical analysis*

The blood glucose concentration was measured using an automated photometric test
and the hexokinase method (Cobas Integra Analyser, Roche). Amylin was measured by an in-
house radioimmunoassay (RIA). In brief, antibodies directed against rat amylin (T-486-6; rabbit
anti-rat-amylin antibodies; kindly provided by T. Pieber, Graz, Austria) were dissolved in
phosphate buffer containing Na₂HPO₄ (50 mMol/L, pH 7.2), aprotinine (1%), EDTA (0.25%),
Triton X 100 (0.2%), fish gelatine (0.1%) and sodium azide (0.02%) (all Sigma). Antibodies
were used at a final dilution of 1:50,000 and rat amylin (Peninsula Laboratories) was used as a
standard. Samples and standards were incubated for 72 h at 4 °C. Radiolabeled amylin (¹²⁵I-
amylin, 9,000-10,000 cpm, Amersham) was added and samples were incubated for 96 h at 4 °C.
Goat anti-rabbit antibodies (Sigma) and polyethylene glycol were added to separate bound from
unbound amylin. Radioactivity was counted by gamma-counter (GAMMAmatic 1, Kontron Analytical).

Insulin and glucagon were measured using commercial RIA kits: insulin using the Linco Porcine Insulin RIA Kit (# PI-12K, Millipore); and glucagon using the ICN Biomedicals kit (# 07152101, MP Biomedicals Europe), both of which were validated prior to use. For the insulin assay, inter- and intra-assay coefficients of variation were 7.0% and 6.5% respectively and the sensitivity was approximately 0.3 µIU/mL (2 pMol/L). Observed-to-expected ratios for serial dilutions (12.5–100%) ranged from 103.2–106.4% (Zini et al., 2008). For the glucagon assay, inter- and intra-assay coefficients of variation were 8.2% and 7.5% respectively and the sensitivity was approximately 50 pg/mL.

Statistical analysis

The area under the curve (AUC) was calculated above the baseline of each individual animal using GraphPad Prism software (GraphPad Inc.) and these values were used to compute the average AUC. All values are given as mean ± standard error of the mean (SEM). The experiments were performed using cross-over design with at least 4 weeks between trials. Statistical tests applied included paired Student's t-tests or repeated measures ANOVA, as appropriate and P values < 0.05 were considered significant.
Results

Pharmacokinetics of rat amylin in cats

Since no data were available relating to the pharmacokinetics of exogenous amylin in cats, we performed a pilot experiment to investigate the time course of plasma amylin levels following the SC administration of a bolus of rat amylin. The rabbit anti-rat-amylin antibodies used do not cross-react with cat amylin (own unpublished observation) and can therefore be used to differentiate between exogenous rat and endogenous cat amylin. Rat amylin was not detected at t = 0 min. The concentration of rat amylin peaked at the first sampling time-point, 5 min after administration and its estimated half-life was approximately 8.4 min. By 30 min after administration, rat amylin was largely cleared from the circulation (Fig. 1).

Intravenous arginine stimulation test

Arginine injection resulted in a slight increase in plasma glucose levels in all groups of approximately 1.5 mMol/L. By 30 min after injection, plasma glucose levels had returned to baseline values. Amylin had no effect on the plasma glucose concentration at any time-point (Fig. 2A). Plasma insulin increased approximately 6-fold following arginine administration (from 7.1 µIU/mL at t = 0 to 45.4 ± 10.2 µIU/mL at t = 7 min). Interestingly, the arginine-induced increase in the plasma insulin concentration was lower in cats previously given amylin (Fig. 2B). Arginine administration also increased plasma glucagon levels and this increase was reduced following amylin administration (Fig. 2C). Amylin treatment reduced the total glucagon secretory response relative to that of control cats, as indicated by the AUC within the first 15 min of its administration (Fig. 2D). Both doses of amylin produced similar effects.
Meal response test

Ingestion of the meal did not result in post-prandial hyperglycaemia, irrespective of treatment so that the plasma glucose concentration was unaffected by amylin administration (Fig. 3A). The post-prandial plasma insulin concentration increased approximately 3-fold relative to fasting levels peaking at 24.3 ± 5.5 µIU/mL immediately when the meal ended and had returned to baseline values within 5 h. The total insulin secretion, as indicated by the AUC, was not affected by amylin administration (data not shown). Plasma glucagon concentrations peaked approximately 1 h after the meal (570 ± 55 pg/mL relative to the baseline value of 265 ± 35 pg/mL) and the total secretion of this hormone over the first hour after the meal, as indicated by the AUC, was significantly reduced by amylin administration (Fig. 3B).

Intravenous glucose tolerance test

The IV administration of glucose increased the plasma glucose concentration in control cats from a baseline of 4.5 ± 0.2 mMol/L to 29.7 ± 4.5 mMol/L in 3 min. Plasma glucose concentrations and the time course of glucose were unaffected by amylin administration. Interestingly, the total insulin output, as indicated by the AUC, was significantly lower in cats that had been given amylin (Fig. 4A). Glucose administration resulted in a time-dependent decrease in plasma glucagon concentrations which was more pronounced in amylin-treated cats relative to controls (Fig. 4B). Both doses of amylin produced similar effects.

Discussion

Amylin plays an important role in the control of nutrient metabolism in rodents and humans, decreasing the excessive post-prandial hyperglucagonaemia observed in DM and
slowing gastric emptying (Edelman and Weyer, 2002; Fineman et al., 2002; Lutz, 2005). Although aggressive insulin treatment can almost normalise mean blood glucose levels in diabetics, enhanced control of post-prandial hyperglycaemia is required for optimal glycaemic homeostasis (Ceriello et al., 2008). This can be achieved by a combination treatment of insulin and amylin, the latter controlling post-prandial glucagon release, an approach that is now used successfully to treat human DM (Thompson et al., 1998, Weyer et al., 2001, Fineman et al., 2002; Ratner et al., 2002; Vella et al., 2002; Hollander et al., 2003, 2004; Maggs et al., 2004).

The results of the present study indicate that similar mechanisms, at least with respect to the control of plasma glucagon, may operate in the cat. Our findings indicate that a single amylin injection lowers plasma glucagon levels under all three test conditions. Blood insulin concentrations were also lowered following amylin administration in the IVAST and IVGTT relative to controls. Intravenous arginine resulted in a slight increase in plasma glucose levels and, as expected, insulin and glucagon concentrations increased (Kitamura et al., 1999). Importantly, the increase in plasma glucagon was significantly reduced by amylin. This was paralleled by a reduction in overall insulin secretion relative to the controls.

Amylin administration also reduced post-prandial glucagon secretion in the MRT. This test mimics normal circumstances indicating that amylin may have therapeutic potential in controlling post-prandial nutrient metabolism in diabetic cats. Interestingly, this feature is considered one of the most significant benefits of the combination insulin-amylin treatment in human diabetics (Young, 2005c). The IVGTT also confirmed amylin’s potency in controlling
plasma glucagon levels. Glucose injection resulted in an expected decrease in the plasma glucagon concentration which was enhanced by amylin administration, and was paralleled by reduced insulin secretion in amylin-treated cats. It is likely that the lower insulin response in the IVAST and IVGTT following amylin administration was secondary to amylin’s effect on glucagon secretion. Although amylin reduces insulin secretion, the low doses of amylin used in the current study are unlikely to have resulted in such an effect (Young, 2005b). However, this issue requires further study. The fact that insulin secretion during the MRT appeared unaffected by amylin administration may be attributable to the smaller increase in the insulin concentration in this test relative to the IVAST and IVGTT. The latter two tests would act as more intense stimulants of insulin release than food intake, so that the short-term influence of amylin on insulin levels in the MRT may not be as easily detected. Given that the pharmacokinetics of rat amylin in cats indicates its plasma half-life is similar to that in other species (Young et al., 1996; Young, 2005a), if amylin were to be used in the treatment of feline diabetes, it would require repeated injection as is the case in humans where two to three meal-contingent injections are recommended Young, 2005c).

A limitation of our study was that, given the effects of amylin on plasma glucagon levels were small, they may not be of great clinical significance. The effects of amylin were not dose-dependent at the two doses we tested and also it must be noted that the cats used in our study were young, healthy and of normal body weight. However, even in such animals, with fully functional endocrine systems, the effect of amylin on the plasma glucagon concentration was found and we suggest this response may be much more pronounced in diabetic cats. Although amylin secretion may be increased initially in diabetics, the later stages of the disease are
characterised by marked hypoamylinaemia (Lutz and Rand, 1996; Young, 2005a,c), so that any
effect of exogenous amylin is likely to be much more pronounced in diabetic cats.

Although the effects of amylin were relatively short-term and occurred at slightly
different time-points in the three tests, we do not consider that this precludes the use of amylin as
an adjunct treatment in diabetes. The current experiment was a ‘proof-of-concept’ study of the
effect of amylin in the cat. The amylin analogue pramlintide, with similar pharmacodynamics to
the amylin preparation used in our study (Young et al., 1996), has been used to successfully treat
T2DM in humans (Fineman et al., 2002; Maggs et al., 2004) and longer acting amylin
compounds are in development. Other limitations to the present study that must be borne in mind
were the relatively small number of cats used and the fact that plasma glucagon concentrations
and not direct secretion were measured. Given that amylin is unlikely to influence renal glucagon
clearance, we suggest that plasma glucagon concentrations reliably reflect pancreatic alpha cell
secretion.

Conclusions

This study provides the first evidence that amylin lowers plasma glucagon concentrations
in the cat. Amylin may therefore have therapeutic potential in diabetic cats, as it has in humans
given that hyperglucagonaemia is a feature of the disease in both species (Tschuor et al., 2006).
The dose of amylin used in this study was comparable to that used in humans and we did not
observe any adverse effects of the treatment. Given the similar pathophysiology of human T2DM
and feline diabetes, and the success in treating human diabetics with a combination of amylin
and insulin, we propose that amylin may have potential use as an adjunct to insulin in the
treatment of diabetic cats, reducing the insulin requirement to control glycaemia.

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antibodies, and Amylin Pharmaceuticals Inc. for supplying the rat amylin used in the study. The
research was funded by the Vetsuisse Faculty.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other
people or organisations that could inappropriately influence or bias the concept of the paper.
References


Figure Legends

Fig. 1
Graph illustrating the time course of the plasma amylin concentration (pMol/L) following the SC injection of rat amylin (5 µg/kg) at t = 0 min (n = 4). The assay only detected exogenous (rat) and not endogenous (cat) amylin.

'y' axis label – Amylin (pMol/L),
'x' axis label – Time after amylin administration (min)

Fig. 2
Graphs illustrating the influence of the subcutaneous administration of amylin (at 5 or 10 µg/kg), or a saline control, on the plasma concentration of glucose (A), insulin (B) and glucagon (C and D) in an arginine stimulation test in six cats fasted for 12 h. Amylin or saline were administered 5 min before arginine (0.2g/kg). The experiment was performed using a cross-over design. (A) Time course of plasma glucose concentration following the administration of arginine, (B) Amylin significantly reduced insulin secretion as assessed by the area under the curve (AUC) during the first 15 min following arginine administration, (C) Amylin significantly reduced plasma glucagon concentrations at 2, 4 and 30 min following arginine administration, (D) Amylin significantly reduced glucagon secretion as assessed by the AUC during the first 15 min following arginine administration. BL = baseline value just prior to administration of amylin or saline. * P < 0.05, **P < 0.01, significantly different from control.

Fig 2A ‘y’ axis label – Glucose (mMol/L), ‘x’ axis label – Time after amylin administration (min)
Fig 2B ‘y’ axis label – Insulin (µIU/mL X 15 min)
Fig 2C ‘y’ axis label – Glucagon (pg/mL), ‘x’ axis label – Time after amylin administration (min)
Fig 2D ‘y’ axis label – Glucagon (µIU/mL X 15 min)
Graphs illustrating the influence of the subcutaneous administration of amylin (5 µg/kg), or a saline control, on the plasma concentration of glucose (A) and glucagon (B) in a meal response test in six cats fasted for 24 h. Amylin or saline were administered 5 min before ingestion of the meal which lasted for approximately 10 min. The experiment was performed using a cross-over design. (A) The post-prandial glucose concentration did not increase above baseline values, (B) Amylin significantly reduced glucagon secretion as assessed by the AUC 1 h after the end of the meal. BL = baseline value just prior to the administration of amylin or saline. * $P < 0.05$, significantly different from control.

**Fig 3A** ‘y’ axis label – Glucose (mMol/L), ‘x’ axis label – Time after end of meal (h)

**Fig 3B** ‘y’ axis label – Glucagon (pg/mL X 1 h)

Graphs illustrating the influence of the subcutaneous administration of amylin (5 or 10 µg/kg), or a saline control, on the plasma concentration of insulin and glucagon in an intravenous glucose tolerance test in six cats fasted for 12 h. Amylin or saline were administered 5 min prior to glucose administration (0.5g/kg). The experiment was performed using a cross-over design. (A) Insulin secretion in the 30 min following glucose administration was lower in amylin-treated cats, (B) Amylin significantly reduced plasma glucagon secretion 3, 7 and 10 min after administration. BL = baseline value just prior to the administration of amylin or saline. * $P < 0.05$, **$P < 0.01$, significantly different from control.

**Fig 4A** ‘y’ axis label – Insulin ($\mu$IU/mL X 30 min)

**Fig 4B** ‘y’ axis label – Glucagon (pg/mL), ‘x’ axis label – Time after glucose administration (min).
Figure 1
Figure 2A

Glucose (mMol/L) over time after arginine administration (min):
- Control
- Amylin 5µg/kg
- Amylin 10µg/kg

Figure 2B

Insulin (µIU/mL x 15 min):
- Control
- Amylin 5µg/kg
- Amylin 10µg/kg
Figure 3A

![Figure 3A](image)

**Glucose (mMol/L)**

<table>
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<th>Time after end of meal (h)</th>
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Figure 3B

![Figure 3B](image)

**Glucagon (pg/mL x 1 h)**

- Control: 11000 pg/mL x 1 h
- Amylin 5µg/kg: 8000 pg/mL x 1 h

*Significant difference
Figure 4A

![Bar chart for Insulin (µIU/mL x 30 min)]

- **control**
- **amylin 5µg/kg**
- **amylin 10µg/kg**

Figure 4B

![Bar chart for Glucagon (pg/mL)]

- **control**
- **amylin 5µg/kg**
- **amylin 10µg/kg**

Time after glucose administration (min)