Short Communication

Amyloidogenesis of feline amylin and plasma levels in cats with diabetes mellitus or pancreatitis


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A B S T R A C T

Amylin is a pancreatic hormone cosecreted along with insulin and involved in pancreatic amyloidosis and β-cell apoptosis in diabetic cats and humans. Amylin is usually elevated in early stages of type 2 diabetes but recently was found to be increased in acute and chronic pancreatitis in humans. Currently, there are little data about feline amylin propensity to fibrillate and no information on circulating levels of this hormone during feline pancreatitis. We compared 4 amylin analogues and found cat amylin to be more prone to amyloid fibrillation than human amylin, the triple-proline analogue pramlintide and rat amylin. We also measured plasma amylin levels in healthy lean cats, diabetic cats, and cats with pancreatitis. Plasma amylin was higher in diabetic cats compared with healthy lean cats (P < 0.001). Interestingly, amylin levels during pancreatitis were higher than those of both lean cats (P < 0.0001) and diabetic cats without pancreatitis (P < 0.005). These data support evidence of feline amylin being more prone to aggregation than human amylin in vitro, which may influence diabetes mellitus progression and β-cell failure in vivo. Furthermore, our data show an increase in amylin levels during feline pancreatitis and the need for future research on the role of this hormone in the pathogenesis of pancreatic inflammation associated to feline diabetes mellitus.

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1. Introduction

Amylin, also known as islet amyloid polypeptide, is a hormone cosecreted with insulin by the pancreatic β-cells [1]. Amylin was first isolated and described from pancreatic amyloid deposits found in both diabetic cats and humans [2,3]. Amylin has several physiologic roles, including control of glucagonemia, bone metabolism, gastric emptying, and satiation [4,5].

The 37 amino acid sequence of amylin is mostly conserved, with the main variations present in the 20 to 29 region of the peptide [6]. Proline-rich variants in the 20 to 29 region (such as rat, hamster, and pramlintide) have been associated with increasing aqueous solubility and decreased propensity for amyloid aggregation, which puts proline-rich variants at lower risk for amyloidogenesis and
formation of small toxic oligomeric agglomerates involved in apoptosis and loss of β-cells [6,7]. Islet amyloidosis seems to be the most common and consistent morphologic feature of the pancreatic islets of humans and cats with type 2 diabetes mellitus [6,8–11].

Humans with subclinical diabetes, that is, under increasing insulin resistance conditions while normoglycemic in the trajectory toward type 2 diabetes [12,13], usually develop an increased production and secretion of insulin and amylin by the pancreatic β-cells [14]. Circulating levels of amylin have also been found elevated in overweight, glucose-intolerant human patients and in those with early type 2 diabetes. On the other hand, the circulating amylin levels decrease with the progression of diabetes [15,16]. Similarly, nondiabetic cats with impaired glucose tolerance have increased circulating concentrations of amylin, while noncomplicated diabetic cats and nondiabetic cats display similar circulating amylin levels. Complicated diabetic cats (ketoacidotic patients) tend to present reduced plasma amylin [17]. The reduction of amylin levels reported in some human and feline diabetic patients has been associated with the loss of β-cells during the progression of the disease [10].

Pancreatitis and diabetes mellitus appear to occur concurrently in many species, including humans and cats [18,19]. Although a causal association has not been proven yet in cats, the concurrence of these 2 diseases has clinical implications for case management and clinical control [20]. Recently, human pancreatitis has been associated with the decrease in β-cell function in a progression rate even higher than in type 2 diabetes mellitus [21]. Previous studies in humans have shown that amylin is increased during acute and chronic pancreatitis [22–24]. Currently, no study has determined if feline pancreatitis can also induce an increase in amylin levels.

Observational studies prospecting amyloid material in vivo have long suggested that proline residues modulate the propensity for amylin analogues to form amyloid aggregations [25]. However, no comparative assays have been reported to date. In fact, the triple proline analogues rodent amylin and the triple-proline (25,28,29Pro) human amylin analogue pramlintide have long been assumed to be nonamyloidogenic and nontoxic in vitro and in vivo, until recent works revealed their propensity in forming amyloid material [26–30].

In the present study, we investigated the amyloid nature of cat amylin compared with other species, exploring the aggregation pattern of cat, human, and murine amylin as well as the amylin analogue pramlintide in vitro. Furthermore, we compared plasma amylin levels between healthy cats, diabetic cats, and cats with pancreatitis.

2. Material and methods

2.1. Reagents

Carboxy-amidated, C2-C7 disulfide bond pramlintide and amylin (feline, human, and murine) (TFA salt, >95% purity) were purchased from Genemed Synthesis Inc (CA). The stock solutions of these peptides were prepared by dissolving in 100% DMSO, aliquoted and stored at −20°C until use. All other reagents were of analytical grade.

2.2. Animals

Twenty-four client-owned cats from private veterinary clinics and from Universidade Federal Fluminense (UFF) Veterinary Hospital (HUVET, Niterói, Brazil) were enrolled in the study.

Lean healthy cats (n = 10) were determined as healthy based on history, physical examination, routine laboratory testing (complete blood count [CBC] and biochemical profile), feline pancreatic lipase (FPL) Snap FPL test, IDEXX Laboratories, Westbrook, ME, and ultrasonographic evaluation. To be included, lean cats had to have a body condition score (BCS) of 3 out of a 5-point scale [31], normal results of FPL, and no abnormalities in the remaining evaluation. Body condition score was always determined by the same examiner (MSMC).

Newly diagnosed diabetic cats (n = 8) were included based on well-established clinical signs and laboratory findings [32]. Briefly, cats were included if they had signs of polyuria, polydipsia, weight loss, and polyphagia and documentation of persistent fasting hyperglycemia and glycosuria. Diabetic cats with ultrasonographic signs of pancreatitis or abnormal levels of FPL were excluded from the study.

Cats with pancreatitis (n = 6) were diagnosed based on clinical signs, CBC, biochemical profile, and abnormal levels of FPL associated with pancreatic ultrasonographic evaluation performed by trained veterinarians. Because clinical signs are nonspecific in cats, any of the following were considered as relevant: anorexia, lethargy, vomiting, diarrhea, and weight loss. As most of the times, hematology and plasma biochemistry findings are unremarkable, CBC and biochemical changes (ex: elevations in liver enzymes and bilirubin) were not essential for the diagnosis. Abnormal levels of FPL and typical ultrasonographic findings (eg: hypoechogenicity of the pancreas, hyperechogenicity of the peripancreatic fat and possible presence of abdominal effusion) were essential for diagnosis.

All blood samples were collected after 12 h of fasting in previously diagnosed cats and using EDTA-containing tubes. Blood (3 mL) was immediately centrifuged at 400 × g for 10 min, the plasma was collected, aliquoted, and stored at −20°C until analysis.

This study was performed according to good clinical practice and has been approved by the ethical committee of our institution (UFF CEUA #403/2013). Owners of cats enrolled in the study signed an informed consent form. Routine laboratory testing and Snap FPL analysis were conducted in the Laboratory of Clinical and Molecular Research, College of Veterinary Medicine, UFF, Rio de Janeiro, Brazil. Amylin measurement was performed atFaculdade de Farmácia, UFRJ (Rio de Janeiro, Brazil).

2.3. In vitro amyloid fibrillation assay

The amyloid fibrillation assays were performed in triplicate in 50 mM Na2HPO4 buffer pH 7.0, with 10 μM peptide (cat, human, or murine amylin; or pramlintide), and continuously monitored by 20 μM Thioflavin T (a specific amyloid fluorescent probe). The assays were performed in a Spectramax M5 (Molecular Devices) plate reader, in 96-
well plates (Corning Cat #3915) at 25°C, with readings performed every 3 min, with excitation and emission set at 440 nm and 482 nm, respectively, and a 475-nm cut-off emission filter.

2.4. Plasma amylin measurement

Amylin levels were determined by ELISA in triplicate using a commercial kit (Amylin, Catalog #EK-017-11, Phoenix Pharmaceuticals, Inc) according to the manufacturer’s protocol. This assay has 100% cross-reactivity with feline amylin according to the manufacturer. We used synthetic feline amylin for the analytical curve.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software). The Shapiro Wilk test was used to evaluate normality of data. Data were considered parametric. To compare data between groups we used one-way ANOVA with Tukey’s post-hoc test. Significance for all tests was set at the $P < 0.05$ level.

3. Results

3.1. Amyloid aggregation pattern

We performed a comparative-controlled evaluation of the 4 amylin analogues (feline, human, pramlintide, and murine) to determine their respective propensities to fibrillate. All tested amylin analogues resulted in amyloid aggregation within 3 d (Fig. 1). Cat amylin showed the highest propensity to aggregate, reaching a maximum ThT signal at about 4 h. Human amylin required about 8 h to reach maximum change in ThT fluorescence. Pramlintide reached maximum amyloid aggregation as expressed by ThT fluorescence in about 12 h. The murine amylin showed the slower aggregation kinetics, taking about 48 h to reach maximum ThT signal transition.

3.2. Circulation levels of amylin in cats

We investigated the plasma levels of amylin in healthy cats, as well as in cats with diabetes mellitus or pancreatitis. Table 1 shows gender, age, and BCS of the 24 owned cats evaluated in this part of the study. The mean age for the lean healthy cats was 7.1 yr ($\pm 2.0$), while cats with pancreatitis were aged an average of 10.2 yr ($\pm 3.3$). Diabetic cats had an average age of 11.3 yr ($\pm 4.6$), and this was significantly different from the healthy group ($P < 0.05$). Newly diabetic cats without pancreatitis showed significantly ($P < 0.005$) increased levels of fasting plasma amylin (19.4 $\pm$ 3.2 pmol/L) compared with healthy lean cats (11.9 $\pm$ 5.2 pmol/L) (Fig. 2). Interestingly, cats with pancreatitis showed an even higher levels of plasma amylin (27.62 $\pm$ 3.0 pmol/L) compared with both diabetic cats ($P < 0.0005$) and lean healthy cats ($P < 0.0001$) (Fig. 2).

4. Discussion

Here we showed that cats with diabetes mellitus or pancreatitis exhibited higher fasting amylinemia compared with healthy animals and animals with early diagnosis of diabetes mellitus. Furthermore, contrary to the previous assumption that proline halts amylin amyloid aggregation [25], feline amylin showed an amyloid fibrillation propensity close to human amylin compared with the less amyloidogenic murine amylin. The isoleucine at position 17 may play a role on this mechanism, given that a single V17I variant in homologous sequences (eg, 17V = panda, seal, bear) confers a highly amyloidogenic character compared with human amylin [33]. This is the first time that a comparative, hierarchical evaluation of the propensity of amyloid aggregation of amylin variants is reported, demonstrating the universal amyloidogenesis nature of amylin, and the fast aggregation process of feline amylin. Given that both diabetic cats and diabetic humans accumulate amylin amyloid material in the pancreas [23], a fact not yet reported for rodents, these results collectively suggest a possible correlation—yet to be further explored—between amyloid propensity, amylinemia, and $\beta$-cell degeneration.

Cats are one of the few species that spontaneously develop a form of DM that closely resembles human type 2 diabetes, including the formation of amyloid deposits derived from amylin aggregation [8,34]. Our results show that feline amylin undergoes in vitro amyloid fibrillation about 2 times faster than human amylin, although the real significance of this fact for in vivo islet amyloidosis during the progression of diabetes in cats remains unknown. Previous studies have associated amyloid deposits with the development of feline diabetes [35,36]. However, a recent study found that the prevalence and extent of islet amyloidosis did not differ between diabetic and matched control cats [37]. Although the latter study hypothesized that microscopically visible insular amyloid is not the primary cause of diabetes in cats [37], the interactions...
between amyloid fibrils and the cell membrane are believed to modulate the calcium influx, which could seriously affect the function of β-cells [38].

Since amylin aggregation may be linked to β-cell death in cats, a precise diagnosis early during the course of the disease might be helpful in preventing or slowing progression of feline diabetes [39]. Indeed, a previous study showed that markers of inflammation and oxidative stress increase early during the development of diabetes in cats [8]. The etiologic classification of diabetes in humans may help with early diagnosis and the categorization of all the subgroups of this disease [40]. Unfortunately, currently there is no established definition for the diagnosis of pre-diabetes or subgroups of diabetes in cats [39].

Our results also showed for the first time that, similarly to humans, feline pancreatitis increases plasma amylin levels, indicating either an overproduction and/or a decrease in its clearance. Previous studies have shown that amylin levels in cats were increased under conditions associated with insulin resistance like obesity and diabetes [17,41]. It is well known that, under insulin resistance, pancreatic β-cells increase the production of both insulin and amylin [42]. Indeed, we reported here that newly diagnosed diabetic cats showed an increase of about 63% in the levels of circulating amylin compared with lean cats. Cats with pancreatitis presented an even higher amylinemia, of about 130% compared with healthy cats. The exact mechanism by which pancreatitis leads to hyperamylinemia remains to be investigated.

Pancreatitis is an acknowledged concurrent disease in diabetic cats [43,44], but diagnosis can be challenging in this species. From a clinical perspective, the present data support the potential of plasma amylin as a surrogate for the early diagnosis of pancreatitis [23] along with other biomarkers, which should involve larger cohorts to increase predictive power and the performance of tests for sensitivity and specificity.

The main limitations of this study are the small number of subjects and the difficulties with diagnosis of pancreatitis. Feline pancreatic lipase was measured using the Snap fPLI assay, which can lead to false positive results. Furthermore, ultrasonographic evaluation was not always performed by the same person in all cats, which could have influenced the exclusion or inclusion of cats. Further studies using a larger study population, better controlled variables and correlating clinical and inflammatory biomarkers could help elucidate the role of amylin in the pathogenesis of pancreatitis and also any clinical relevance for the diagnose of the disease.

5. Conclusions

The proline-stabilizing principle should not be taken as a deterministic feature when isolated from a broader sequence and structural context. Moreover, the plasma levels of amylin constitute a possible indication for pancreatitis in cats compared with healthy lean and diabetic cats.

CRediT authorship contribution statement

**L. Jotha-Mattos**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - review & editing. **A.B. Vieira**: Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing - review & editing. **M. da S.M. Castelo**: Investigation, Writing - review & editing. **A.S. de M. Queiroz**: Investigation, Writing - review & editing. **H.J.M. de Souza**: Investigation, Writing - review & editing. **N.X. de Alencar**: Data curation, Investigation, Writing - review & editing. **L.M.T.R. Lima**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation,
Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

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