The anorexic hormone Peptide YY$_{3-36}$ is rapidly metabolized to inactive Peptide YY$_{3-34}$ in vivo

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**Abstract**
Peptide YY (PYY) is a 36 amino acid peptide hormone released from enteroendocrine cells. An N-terminally degraded metabolite, PYY$_{3-36}$, has anorexigenic effects, which makes the PYY system a target for obesity treatment. However, little is known about the kinetics and degradation products of PYY. A related peptide, Neuropeptide Y (NPY), may be degraded from the C-terminus. We therefore investigated PYY degradation after in vitro incubations in porcine plasma and blood and in vivo by infusing PYY$_{3-36}$ into multicatheterized pigs ($n=7$) (2 pmol/kg/min). Plasma samples were analyzed by region-specific radioimmunoassays (RIA) and HPLC analysis. A metabolite, corresponding to PYY$_{3-34}$ was formed after incubation in plasma and blood and during the infusion study. When taking the C-terminal degradation into account, the half-life ($T_{1/2}$) of PYY in blood and plasma amounted to 3.4 ± 0.2 and 6.2 ± 0.2 h, respectively. After PYY$_{3-36}$ infusion in pigs, the peptide was degraded with a $T_{1/2}$ of 3.6 ± 0.5 min. Significant extraction (20.5 ± 8.0%) compatible with glomerular filtration was observed across the kidneys and significant C-terminal degradation (26.5 ± 4.8%) was observed across the liver. Net balances across the hind limb, splanchnic bed, and lungs were not significantly different from zero. PYY$_{3-34}$ was unable to activate the Y2 receptor in a transfected cell line. In conclusion, PYY$_{3-34}$ is extensively degraded to PYY$_{3-36}$ in the pig, a degradation that renders the peptide inactive on the Y2 receptor. Currently used assays are unlikely to be able to detect this degradation and therefore measure falsely elevated levels of PYY$_{3-36}$, leading to underestimation of its physiological effects.

**Introduction**
Peptide YY (PYY) is a peptide hormone released from a subpopulation of enteroendocrine cells following food intake (Adrian et al. 1985). PYY influences appetite, gastric motility, and water and electrolyte absorption. PYY belongs to a small family of peptides, including PYY, NPY, and Pancreatic Polypeptide (PP). These are all 36 amino acid, C-terminally amidated peptides with high contents of tyrosine, proline, and arginine residues. Their tertiary structure is characterized by a high helical content plus a hairpin structure, often designated “the PP-fold” (Tatemoto 1982; Glover et al. 1984). The family, including PYY binds to and activates Y-receptors. The intact peptide, PYY$_{1-36}$, acts on Y1, Y2, and Y5 receptors, while the N-terminal truncated metabolite, PYY$_{3-36}$, acts on the Y2 receptor. Only the Y2 receptor mediate anorexigenic effects, and therefore only PYY$_{3-36}$, but not PYY$_{1-36}$, has attracted interest as a possible drug candidate for obesity treatment (Abbott et al. 1043; Keire et al. 2002).

Pep tide YY is synthesized and stored as the 36 amino acid peptide, PYY$_{1-36}$. After its release, the enzyme dipetidyl peptidase-4 (DPP-4; EC 3.4.14.5), cleaves off two N-terminal residues leading to the formation of the...
Anorexigenic PYY\textsubscript{3\--36} (Eberlein et al. 1989; Medeiros and Turner 1994). PYY\textsubscript{3\--36} is thought to make up 40–55% of total circulating PYY in humans (Grandt et al. 1994), and the formation of PYY\textsubscript{3\--36} can be almost totally blocked by administration of a DPP-4 inhibitor (Aaboe et al. 2010).

Besides hydrolysis by DPP-4, in vitro studies showed that PYY can be degraded by aminopeptidase P, neutral endopeptidase 24.11 (NEP 24.11), and meprin \( \beta \) (Fig. 1) (Medeiros and Turner 1994; Addison et al. 2011). However, the importance of these pathways for the metabolism of circulating PYY is unknown. Being a C-terminally amidated peptide, PYY\textsubscript{3\--36} is usually considered relatively protected from C-terminal degradation. However, Abid et al. showed that another peptide from the PP family, NPY, is degraded from C-terminal degradation. However, Abid et al. showed that another peptide from the PP family, NPY, is degraded to NPY\textsubscript{1\--34} in human serum (Abid et al. 2009). In another study, NPY\textsubscript{1\--32}, NPY\textsubscript{1\--34}, and NPY\textsubscript{1\--35} were found to be the main metabolites after extensive human plasma incubation (Khan et al. 2007). As PYY and NPY exhibit high homology (70%) and the residues 32–36 are identical, PYY might also be subject to C-terminal degradation.

The measurement of PYY in plasma samples is commonly carried out by RIA or ELISA, using either a specific PYY\textsubscript{3\--36} antibody binding to the truncated N-terminal, or a “side-viewing” antibody which detects both PYY\textsubscript{1\--36} and PYY\textsubscript{3\--36}. These assays provide no information about the C-terminus, and the amount of active hormone may therefore be overestimated if a significant C-terminal degradation occurs. NPY being metabolized to NPY\textsubscript{3\--35} and NPY\textsubscript{3\--34} (Khan et al. 2007; Abid et al. 2009) led us to suspect that PYY\textsubscript{3\--34} might be formed in vivo from exogenous as well as endogenous PYY. This study was designed to examine this hypothesis.

Materials and Methods

This study conformed to the Danish legislation governing animal experimentation, and permission was granted from the National Superintendence for Experimental Animals.

Anesthetized pigs – exogenous PYY – infusion study

Seven female pigs of the LYD/LYH strain weighing approximately 30 kg were used. Animals were fasted for 16 h but allowed access to drinking water. After premedication with Ketamine (Ketaminol, 10 mg/kg), the animals were anesthetized with \( \alpha \)-chloralose (Sigma C0128, 100 mg/kg) and ventilated with intermittent positive pressure with \( \text{N}_2\text{O}/\text{O}_2 \). Vascular catheters were inserted into the pulmonary and a carotid arteries, and into the left femoral, the portal, the hepatic, the left renal, and an ear vein, as described previously (Deacon et al. 1996). After surgery, animals were heparinized and left undisturbed for 30 min.

Synthetic porcine PYY\textsubscript{3\--36} (Bachem H-6042) was dissolved in phosphate buffer with 2% Human serum albumin (HSA, Calbiochem, Merck Millipore, Darmstadt, Germany) and at \( t=0 \) a bolus injection (5 pmol/kg) was given in the right ear vein immediately followed up by the infusion of PYY\textsubscript{3\--36} into the right ear vein at a rate of 2 pmol/min/kg using a precision syringe pump. Arterial blood samples were drawn from the carotid artery at \(-15\), \(-10\), 0, 10, 20, 25, 30 min. Between time points 30 and 40 min, two duplicate sets of samples were taken simultaneously from all vascular catheters (carotid artery, pulmonary artery and the femoral, the portal, the hepatic, and the left renal vein). Blood samples were collected into chilled tubes containing EDTA (7.4 mmol/L) and aprotinin (500 KIU/mL), and kept on ice until centrifugation within 20 min. Plasma samples were stored at \(-20^\circ \text{C} \) until RIA analysis. The PYY infusion was stopped after 45 min and additional arterial blood samples were taken after 2, 5, 10, 15, 20, 30, 45, 60, 85, 115, and 140 min. At \( t=140 \) min, 100 pmol/kg PYY\textsubscript{3\--36} was injected and 15 min later blood was sampled for HPLC analysis.

Anesthetized pigs – endogenous PYY – neuromedin C

Nine female pigs of the LYD/LYH strain weighing approximately 30 kg were anesthetized as described above and catheters placed in the carotid artery and the portal vein. Neuromedin C (120 nmol, Bachem H-3120) was given as a bolus injection at \( t=0 \) min, and blood was collected from the carotid artery at \(-15\), \(-5\), 0, 2, 5, 10, 15, 20, 30, 45, and 60 min.

In vitro degradation of PYY in plasma and blood

Porcine PYY\textsubscript{1\--36} (p-PYY\textsubscript{1\--36}) (Bachem H-4505) was incubated in heparinized plasma and whole blood at 37°C for 24 h with/– EDTA (10 mmol/L) and +/– aprotinin (1000 KIE/mL). Samples were taken out regularly and mixed with EDTA, aprotinin and the DPP-4 inhibitor valine pyrrolidide (0.01 mmol/L, final concentration) and frozen instantly, the blood samples after centrifugation, and separation of plasma.

Hormonal analysis

Plasma samples were analyzed for PYY using two in-house RIAs. Total PYY was measured with a “side-viewing”
antibody (T-4093, Bachem), which recognizes PYY$_{1-36}$ and PYY$_{1-34}$ equally well. As tracer, $^{125}$I labeled p-PYY$_{1-36}$ was used (NEX240, Perkin Elmer). The C-terminally truncated metabolite was measured using a new antiserum (code no. 2940) raised in white rabbits against residues PYY$_{23-34}$ coupled to keyhole limpet hemocyanin. Synthetic porcine PYY$_{3-34}$ (Genscript, Piscataway, NJ) was used as standard and the tracer was synthetic human PYY$_{3-34}$, $^{125}$I labeled using stoichiometric chloramine-T method. The assay detects both PYY$_{1}$ labeled using stoichiometric chloramine-T method. The assay detects both PYY$_{1}$ and PYY$_{1/3}$ and shows negligible cross-reaction with p-PYY$_{1-36}$. Assay buffer for both assays was 0.1M tris, pH 8.5, containing 0.2% (wt/vol) HSA, 20 mmol/L EDTA, and 0.6 mmol/L thiomersal (Sigma, St. Louis, MO). Free and bound moieties were separated with plasmacoated charcoal (E. Merck, Darmstadt, Germany). Before PYY measurements plasma was extracted with 70% ethanol (vol/vol, final concentration) to avoid unspecfic interference from plasma proteins. Both assays have detection limits below 3 pmol/L (3*standard deviation of the blank) and intraassay variations below 6%.

**High-performance liquid chromatography**

Plasma sampled 20 min after the 100 pmol/kg PYY$_{3-36}$ bolus injection was subjected to solid phase extraction (SepPak® Plus tC18, Waters, Milford, MA) and reconstituted in phase A (10% acetonitrile in H$_2$O, 0.1% trifluoroacetic acid [TFA]). Plasma was spiked with 0.64 pmol p-PYY$_{3-36}$ or p-PYY$_{1-36}$ and loaded onto a HPLC system (Akta Purifier 900, Amersham Biosciences, Brondby, Denmark) equipped with a Vydac C18 Denali column (238DE54). PYY$_{3-36}$ and PYY$_{3-34}$ were separated by gradient elution (23 to 28% B in 20 min, Phase A: 10% acetonitrile in H$_2$O, 0.1% TFA, Phase B: 10% H$_2$O in acetonitrile, 0.1% TFA). The flow was set to 1 mL/min and fractions were collected every 12 sec.

**Transfections and tissue culture**

COS-7 cells were grown at 10% CO$_2$ and 37°C in Dulbecos modified Eagles medium with GlutaMAX (Invitrogen, Slangerup, Denmark) added 10% fetal bovine serum, 180 U/mL penicillin and 45 μg/mL streptomycin (Pen-Strep, Thermo Fischer, Slangerup, Denmark). Cotransfection of the hNPY receptor 2 (Y2 receptor) and Gqi4myr was performed using the calcium phosphate precipitation method, as described by Kissow et al. (2012).

**Phosphatidylinositol assay**

The cotransfected COS-7 cells were incubated for 24 h with 5 μCi/mL [myo$^3$-H]inositol in growth medium and the assay was carried out as described by Rosenkilde et al. (2005). Three independent experiments with duplicate measurements were performed using increasing concentrations of porcine PYY$_{3-36}$ and porcine PYY$_{3-34}$ (from 10 pmol/L to 1 μmol/L). The dose-response curves were fitted using GraphPad Prism, LaJolla, CA.

**Calculations**

Peptide YY$_{3-36}$ concentrations were estimated by subtracting PYY$_{3-34}$ concentrations from total PYY concentrations.

Half-life of PYY in blood and plasma during in vitro incubation was determined from estimated PYY$_{3-36}$ concentrations using the one phase decay model of Prism. For each animal, organ extractions of PYY were calculated from arteriovenous concentration differences, as described in detail previously (Deacon et al. 1996).

The in vivo plasma half-life ($T\frac{1}{2}$) of PYY was determined from the carotid plasma concentrations during the elimination phase (after subtraction of basal arterial concentrations) using the one phase decay model of Prism. The metabolic clearance rate was determined from the actual infusion rate divided by the carotid plasma concentration during steady state (mean of plasma concentrations at $t = 33, 39$ and 45 min, basal values subtracted).

**Statistics**

Data are expressed as mean ± SEM and analyzed by $t$-test for paired data or one-way ANOVA as appropriate. Differences resulting in $P < 0.05$ were considered significant. Statistical analyses were carried out using GraphPad Prism, version 5.04 for Windows, GraphPad Software).

**Results**

**Degradation in vitro**

Peptide YY$_{3-36}$ was degraded in blood and plasma in vitro with $T\frac{1}{2}$ of 3.4 ± 0.2 and 6.2 ± 0.6 h, respectively. In blood, an inexplicable rise in total PYY occurred during the first hour of incubation, and the first two time points were therefore excluded in the determination of half-life. When aprotinin was added to the incubation mixture, the half-life was significantly prolonged to 10.8 ± 0.8 and 11.9 ± 0.7 h, respectively ($P < 0.05$). After addition of EDTA, the degradation no longer followed first-order kinetics, and after 24 h more than 80% of PYY was preserved (Fig. 2).

**Metabolism of exogenous PYY$_{3-36}$ in vivo**

The PYY$_{3-36}$ infusions resulted in a steady state concentration of 228 ± 21 pmol/L total PYY. After the infusion
was stopped, PYY was eliminated by first-order kinetics with a $T_{1/2}$ of 7.3 ± 0.8 min. When corrected for C-terminal degradation (i.e., subtraction of PYY$_{3-34}$ values from total PYY values), the $T_{1/2}$ was significantly shorter and amounted to 3.6 ± 0.5 min ($P < 0.05$) (Fig. 3). Metabolic clearance rate was 12.0 ± 1.2 mL/min/kg calculated from the results of the total PYY RIA but after correction for the C-terminal degradation, the metabolic clearance rate was significantly greater ($P < 0.01$), 28.1 ± 3.7 mL/min/kg.

Significant extraction (20.5 ± 8.0%) of PYY$_{3-36}$ (calculated as PYY$_{3-34}$ subtracted from total PYY), compatible with glomerular filtration was observed across the kidneys, and significant extraction due to C-terminal degradation (26.5 ± 4.8%) was observed across the liver. Net balances across the hind limb, splanchnic bed, and lungs were not significantly different from zero (Fig. 4). PYY$_{3-34}$ was also extracted across the kidneys with a ratio compatible with glomerular filtration, but no significant changes were found across the hind limb, splanchnic bed and lungs. Across the liver, a net formation (31.4 ± 1.8%) was found (Fig. 4).

**Separation of plasma PYY moieties by HPLC**

After HPLC separation all fractions were subjected to both total PYY and PYY$_{3-34}$ RIA analysis. The total PYY RIA showed three peaks of which two were identified as...
PYY\textsubscript{3-34} and PYY\textsubscript{3-36}. The identification was performed by analysis of the same plasma sample, spiked with the three different PYY moieties (p-PYY\textsubscript{1-36}, p-PYY\textsubscript{3-36}, and p-PYY\textsubscript{3-34}). There was no immunoreactivity at the position of p-PYY\textsubscript{1-36}. The total PYY assay detected an early peak which did not correspond to any of the spike peptides. The PYY\textsubscript{3-34} assay only recognized the PYY\textsubscript{3-34} peak (Fig. 5).

Endogenous PYY is degraded to PYY\textsubscript{3-34}

After stimulation of PYY secretion with neumedin C, plasma concentrations of total PYY, PYY\textsubscript{3-36}, and PYY\textsubscript{3-34} were significantly higher than basal levels. Neumedin C stimulated PYY release to a peak concentration of total PYY of 81.1 \pm 10.5 pmol/L and when corrected for C-terminal degradation to PYY\textsubscript{1/3-34} the peak concentration was 59.8 \pm 10.0 pmol/L (Fig. 6).

PYY\textsubscript{3-34} does not activate the Y2 receptor

In COS-7 cells transiently transfected with the human Y2 receptor and the chimeric G-protein Gqi4myr, we found no phospholipase C activation upon stimulation with PYY\textsubscript{3-34} (Fig. 7). Porcine PYY\textsubscript{3-36} signaled through the receptor with potency and efficacy similar to human PYY\textsubscript{3-36} having EC\textsubscript{50} values of 3.9 nmol/L (Fig. 7) and 3.5 nmol/L, respectively.

Discussion

Recent research has shown that a certain amount of NPY\textsubscript{3-36} is degraded to NPY\textsubscript{3-35} when incubated in human plasma (Abid et al. 2009). NPY and PYY are highly homologous and it might therefore be expected that PYY is also degraded from the C-terminus.

In the present investigation, two different assays were used to detect PYY. The antibody used in the assay for...
total PYY was raised against full-length PYY and detects both PYY1-36 and PYY1-34. The antiserum used in the PYY1-34 assay was raised against PYY23-34 and cross-reacts with NPY1-34, but exhibits less than 1% cross-reaction with PYY1-36. The cross-reactivity with NPY was not considered a problem, since the concentrations of PYY in the in vitro incubation study were expected to exceed that of (endogenous) NPY many times and in the infusion study, the shape of the PYY3-34 curve follows that of the infused PYY3-36. When HPLC fractions were analyzed with the two assays, the PYY3-34 assay only detected the PYY3-34 peak confirming the selectivity of the assay. The total PYY assay detected all the expected peaks (identified by spiking of plasma), but an additional peak was also found. This peak has not been identified, but may reflect another cleavage product of PYY, indicating that the half-life of intact PYY3-36 might be shorter than 3.6 ± 0.5 min. We estimated the level of PYY3-36 by subtracting PYY3-34 from total PYY concentrations. The resulting concentration of such a subtraction is subject to some uncertainty, as the variance of both assays influences the result. Furthermore, it cannot be excluded that the side-viewing antibody binds to other C-terminal degraded moieties that are not recognized by the C-terminal specific assay and vice versa. The only way to overcome this problem is to use a sandwich ELISA with C-terminal and N-terminal specific antibodies, but to our best knowledge assays like this do not exist. Abid et al. found that NPY is degraded to NPY3-35, but only detected trace amounts of NPY3-34. In contrast to the cleavage products found by Abid et al., and in agreement with the data presented here on PYY degradation, Khan et al. (2007) incubated NPY (fluorescently labeled at residue 1) in human plasma and found that after 24 h 50% of the peptide was degraded and that the main cleavage products were NPY1-32 and NPY1-34. When NPY was labeled at residue 4 instead, the main cleavage product was NPY1-35, indicating that the N-terminal structure influences C-terminal degradation.

Meprin β is reported to hydrolyze PYY between residues 10 and 11 and there are several cleavage sites for NEP 24.11 in PYY. Both enzymes are metalloendoproteases and inhibited by EDTA consistent with the attenuation of total PYY breakdown in the incubation study.

The incubation study also revealed that the breakdown of PYY in both blood and plasma is slow (half-life 3.4 ± 0.2 and 6.2 ± 0.6 h, respectively) and even slower when adding EDTA. Thus, soluble plasma enzymes do not appear to contribute heavily to the degradation of PYY in vivo. Furthermore, the data demonstrate that the risk of PYY degradation after blood sampling in EDTA tubes is small.

The present investigation shows that both total and C-terminally degraded PYY are eliminated by the kidney with an extraction ratio amounting to about 20%, which would be compatible with a loss by glomerular filtration (GFR) assuming that GFR would represent 20% of total renal plasma flow (RPF). GFR has been shown to represent approximately 24–27% of RPF in pigs of same strain and size (Link et al. 1985; Hansen et al. 1998). Thus, glomerular filtration may be the main mechanism for renal elimination of PYY in pigs.

In our study, MCR calculated on the basis of plasma profiles obtained by using the total PYY assay (12.0 ± 1.2 mL/min/kg) amounts to about 4–5 times more than what can be explained by glomerular filtration. There was no significant extraction across the other organs, and degradation by soluble enzymes in blood cannot explain the elimination, as the half-life found after in vitro incubation in blood was 3.4 ± 0.2 h.

Interestingly, our in vivo studies showed that PYY is subjected to considerable C-terminal degradation in the liver, and that this degradation is responsible for a hepatic extraction rate of PYY3-36 of 26.5 ± 4.8%. This reveals that the liver and the kidneys are major sites for inactivation/elimination of PYY. An earlier study by Beckh et al., employing in situ perfusion of the rat liver, has shown a lower hepatic extraction of PYY (up to 10% in concentrations up to 500 pmol/L) (Beckh et al. 1992), but the PYY assay they used most likely could not detect C-terminal...
changes; this would be in accordance with our results with the total PYY assay, where no significant hepatic extraction was detected.

Several studies have shown that the C-terminus is crucial for Y2 receptor activation (Beck-Sickinger et al. 1994; Beck-Sickinger and Jung 1995), and that the loss of the two C-terminal residues most likely interrupt the interaction between PYY and the Y2 receptor.

In accordance with these observations, we found no activity of porcine PYY3–34 on the human Y2 receptor.

**Conclusion**

In this study, we describe a significant formation of a C-terminally truncated PYY metabolite, PYY3–34 as part of the in vivo metabolism of PYY. The currently used PYY assays are unlikely to discriminate between this and intact PYY. Measurements with these assays are therefore likely to overestimate the level of physiologically active hormone, as PYY3–34 is not active on the Y2 receptor. Further studies will be required to investigate whether or not the metabolite has other activities.

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**Conflict of Interest**

The authors declare no financial or other conflicts of interest.

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