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Tetra-Hydroxylated Bile Acid in Healthy and Diabetic Cats, and its Effect on Adipocyte Size and Insulin Sensitivity in Healthy Cats

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Abstract

Obesity leads to insulin resistance (IR) and is a major risk factor for the development of diabetes mellitus in cats. Easy prevention of IR and diabetes mellitus would be avoiding obesity. However, reliable long-term strategies are currently lacking. Recently, retinoid-related orphan receptor gamma (RORγ) has been identified as an important transcription factor in the development of large insulin-resistant adipocytes. RORγ can be inhibited by its ligand tetra-hydroxylated bile acid (THBA). Oral supplementation of THBA reduces adipocyte size, improves insulin sensitivity and prevents hyperglycemia in obese mice. The knowledge about THBA and its effect in feline adipose tissue is missing. In the present study THBA was determined in healthy and diabetic cats, and possible side effects of THBA, and the effects of THBA supplementation on adipocyte size and insulin sensitivity were investigated in healthy cats.

Thirteen healthy and 13 diabetic cats were used for determination of serum THBA and six healthy normal-weight cats were included in a feeding trial. THBA was detectable in all samples, however there was no difference between healthy and diabetic cats. Oral THBA supplementation for eight weeks was well tolerated by all cats. It significantly reduced adipocyte size and mRNA expression of matrix metalloproteinase 3, interleukin 6 and tumor necrosis factor α in subcutaneous adipocytes, while mRNA expression of adiponectin significantly increased. However, THBA did not influence fasting blood glucose and cats` response to acute insulin administration. Based on these results, THBA is considered safe for use in cats. It promotes the development of small insulin sensitive adipocyte and might prevent feline obesity-induced IR. Further studies are needed to evaluate effect of THBA in obese cats.

Keywords

Bile acid, insulin sensitivity, adipocyte, tetra-hydroxylated bile acid, diabetes mellitus, retinoid related orphan receptor gamma, cat

Kommentiert [EZ1]: …I would rather write insulin sensitivity, if this is what you mean.

1. Introduction

Obesity is major risk factor for the development of insulin resistance (IR) and diabetes mellitus in cats (1,2). Therefore, treatment and prevention of obesity are important goals in managing diabetic cats (3). Weight loss programs are challenging and often miss long-term success (4). Approved drugs for treatment of feline obesity are lacking (5). Some glucagon-like peptide (GLP)-1 analogs were recently shown to lead to short-term weight-loss effect in healthy cats (6,7). However, their long-term effect on body weight in obese non-diabetic and diabetic cats is unknown.

The pathophysiology of obesity-induced IR is complex. Among several factors, IR is influenced by two main mechanisms of the white adipose tissue to increase its mass: hyperplasia (de novo synthesis of adipocytes) and hypertrophy (enlargement of adipocytes) (8,9). Large hypertrophic adipocytes develop IR, while small hyperplastic cells retain normal insulin sensitivity and function. The balance between hypertrophy and hyperplasia considerably affects the metabolism and insulin sensitivity in obese mice and humans. White adipose tissue expansion by hypertrophy is associated with development of IR (9-13).

An important regulator of adipogenesis and insulin sensitivity in mice and humans is retinoic acid-related orphan receptor γ (RORγ) (14,15). The main isoform of RORγ negatively affects adipocyte differentiation through expression of its target gene matrix metalloproteinase 3 (MMP3) and encourages the development of large insulin resistant adipocytes. Obese RORγ deficient mice have pronounced increase of adipocyte hyperplasia (i.e., small adipocytes) and are protected from obesity-induced IR. Additionally, in obese humans increased expression of RORγ is associated with large adipocyte size and insulin resistance (14,16). Recently, tetra-hydroxylated bile acid (THBA) was identified as a novel inverse agonist for RORγ in mice (16). THBA is a naturally occurring hydrophilic bile acid. It is so far described in mice and humans and may have choleretic and hepatoprotective effects (17-19). In a feeding trial supplementation of synthetic THBA together with pure high fat diet for six weeks reduced adipocyte size in murine subcutaneous and epidydimal adipose tissue and protected mice from the development of diet-induced IR. Moreover, oral supplementation of THBA for 16 weeks reduced adipocyte size and fasted blood glucose in mice with induced IR compared to the control group (16). These preliminary results suggest, that THBA might be used for prevention and treatment of obesity-induced IR in affected individuals.

The knowledge about endogenous THBA and the effect of THBA supplementation on RORγ and IR in cats is lacking. Therefore, we decided to perform a study with the following aims:

Kommentiert [EZ2]: In the manuscript there is a lot of "feline". In the past we tended to write "in cats" instead of "feline".
Fine with me if you keep "feline".

Kommentiert [EZ3]: I would not write that cats have T2DM.
I would rather add a short sentence to say that DM in cats shares many similarities to type 2 DM in humans, including other than obesity induced IR, islet amyloid…

(1) determination of endogenous THBA concentrations in healthy and diabetic cats, (2) evaluation of side effects of synthetic THBA supplementation in healthy normal weight cats, (3) investigation of the effect of synthetic THBA on adipocyte size, fasting glucose and insulin tolerance in healthy normal weight cats. Our hypotheses were (1) healthy cats have higher THBA blood level compared to diabetic cats, (2) synthetic THBA is well tolerated and safe for use in cats, (3) oral application of synthetic THBA for eight weeks encourages formation of small subcutaneous adipocytes, reduces fasting blood glucose and improves insulin tolerance in healthy normal weight cats.

2. Material & Methods

The study design was approved by the Cantonal Veterinary Office of Zurich, Switzerland (permission number: 03/2015).

2.1 Endogenous THBA in healthy and diabetic cats

2.1.1 Cats

Twenty-six cats were included. Thirteen cats were healthy (seven client-owned cats and six cats from the feeding trial, see 2.2). The cats were considered healthy according to an unremarkable physical examination and blood work (hematology, biochemical profile). Among them were 12 European shorthair and one British shorthair. Eleven cats were male castrated and two were female spayed. Median age was 6.4 years (range, 3.5-10.5), median body weight was 5.1 kg (range, 4.0-6.5) and median body condition score (BCS) was six (range, 4-7) on the nine points scale (20). The other 13 cats were diagnosed with diabetes mellitus and treated for an overage period of one year. Diagnosis was based on clinical signs (e.g., polyuria/polydipsia, weight loss, polyphagia), hyperglycemia, increased serum fructosamine and glycosuria. Four diabetic cats were additionally diagnosed with diabetic ketoacidosis and one cat with diabetic ketosis. Diabetic group included eleven European shorthair cats, one Burmese and one Persian. Seven cats were male castrated and six were female spayed. Median age was 12.2 years (range, 8.4-16.6), median body weight was 5.9 kg (range, 3.3-8.0) and median BCS was seven on the nine points scale (range, 3.0-9.0). Diabetic cats were treated with insulin glargine (Lantus, Aventis) or protamine zinc insulin, (ProZinc, Boehringer Ingelheim).

117 2.1.2 Blood sampling and THBA measurement
118
119 Blood samples were collected at the Clinic for Small Animal Internal Medicine, Vetsuisse
120 Faculty, University of Zurich, Switzerland during routine checkup or hospitalization. All
121 samples were analyzed by an in-house laboratory immediately after collection (hematology,
122 biochemical profile, fructosamine). Remaining serum was stored at -80°C for batch analysis.
123 Serum THBA was measured as follows. For preparation of standard dilution stock solution
124 (0.025 mg/mL tauro 1β-THBA in methanol-water (1:4) was diluted 1:1, 1:2, 1:3 and 1:4 with
125 methanol-water (1:4). Bond Elute C16 cartridges (particle size of 40 µM, Agilent) were used
126 to enrich THBA by solid phase extraction. They were conditioned by sequential washes with
127 2mL of methanol, 1mL of chloroform and 1 mL of water. Then 300 µL serum sample were
128 loaded and the cartridges were washed with 1mL water. Bile acids were eluted with 1mL of
129 methanol, dried and resuspended in 100 µL of methanol-water (1:4). 20µL of each sample
130 were analyzed by Liquid Chromatography Mass Spectrometry on a stationary Phase (Column:
131 Agilent Eclipse C18, 30 x 3mm, 3.5 µm Mob.; Phase: 0.6ml/min; Gradient: Linear). Internal
132 standard (4D tauro cholic acid) was used to normalize samples for material loss during bile
133 acid purification. Normalized counts were plotted against the amount of tauro 1β-THBA in
134 standard dilutions. Thereafter linear regression was performed and amount of THBA in feline
135 serum samples was calculated from the axis intercept.
136
137 2.2 Feeding trial
138
139 2.2.1 Cats
140
141 Six healthy purpose-bred male castrated domestic short hair cats were used for the feeding
142 trial. Median age was 4.7 years (range, 4.4-4.7), median weight was 5.0 kg (range, 4.2-5.7)
143 and median body condition score was five on the nine-point scale (range, 4.0-6.0).
144
145 2.2.2 Design of feeding trial
146
147 Each cat was fed 5 mg/kg of synthetic THBA (16) in a small amount of canned food (Hill's
148 a/d™, Hill's Pet Nutrition) once a day in the morning for a period of eight weeks.
149 Additionally, cats were fed a commercial dry food (Hill's™ Science Diet™ Adult Optimal
150 Care, Hill's Pet Nutrition) to maintain body weight. All cats were evaluated three or four

times daily for general condition and possible side effects of THBA. At week zero and week eight, cats underwent a complete physical examination and were intramuscularly sedated with 0.1 mg/kg of midazolam (Dormicum, Roche AG) and 4 mg/kg of S-ketamine (Keta-S, Graeub). Thereafter general anesthesia was intravenously induced and maintained with alfaxalone (Alfaxan, Jurox) for insulin tolerance test (ITT) and sampling of subcutaneous adipose tissue. All cats were fasted for 10-12 h before sedation.

2.2.3 Blood analysis and ITT

Blood samples were taken from the jugular vein immediately after sedation and analyzed as described above (see 2.1). Feline insulin was measured by Mercodia Feline Insulin Elisa (10-1233-01 Mercodia AB Uppsala Sweden) according to manufacturer's recommendations. ITT was performed under general anesthesia (see 2.2.2). First fasting blood glucose was measured twice (interval of five minutes) using a portable blood glucose meter (AlphaTRAK, Zoetis). Then ITT was started by intravenous injection of 0.1 U/kg insulin aspart (NovoRapid, 100 U/mL, Novo Nordisk Pharma) (21). Capillary blood glucose was measured consecutively every five minutes using the above-mentioned portable glucometer. The test was terminated by intravenous application of 0.5 mL/kg of 50% glucose solution diluted with 0.9% saline, when the blood glucose dropped below 2.0 mmol/L or when the blood glucose declined by half of the initial value. The halftime of the blood glucose ($t_{½}$) was recorded and the rate constant for the disappearance of glucose (K_{ITT}) was calculated according to the formula $K_{ITT} = 0.693/t_{1/2} \times 100$ (21).

2.2.4 Adipose tissue

Subcutaneous adipose tissue samples were taken in the umbilical area by a 4 mm punch biopsy. One part of each sample was fixed in 10% formalin solution for 24 hours and stored thereafter in 65% EtOH. The slides for histological analysis were prepared by paraffin embedding and sectioning to 10 µm slices. Slices were stained with H&E and analyzed for adipocyte size using computer image analysis (CellProfilerTM, Broad Institute of Harvard and MIT) (14,22). The second part of each fat tissue sample was immediately frozen at -80°C for RNA isolation. Total RNA was isolated (Trizol, Invitrogen), DNAse treated (Qiagen, USA), quantified with aid of NanoDrop (Implen, Germany) and transcribed into cDNA (High Capacity cDNA Reverse transcription kit, Applied Biosystems) as described previously (14).

Expression of RORγ, MMP3, IL6, TNFα, adiponectin and leptin were determined by quantitative real-time PCR (StepOnePlus, Applied Biosystems) using SybrGreen (Invitrogen) according to the manufacturer's recommendations (14). Primer sequences are listed in Table 1. Relative mRNA levels were normalized to β-Actin which was used as housekeeping gene.

2.3 Statistical analysis

Statistical evaluation was performed by IMB® SPSS® Statistics 25. Shapiro-Wilk test, Mann-Whithey U test and Wilcoxon test were used. The differences were considered significant at $P<0.05$.

3. Results

3.1 Endogenous THBA in healthy and diabetic cats.

Endogenous THBA could be detected in all serum samples. Median serum THBA was 0.56 µmol/ml (range, 0.17-0.79) in healthy cats and 0.49 µmol/ml (range, 0.25-1.60) in diabetic cats. There were no significant differences (P=0.62) between both groups. Serum THBA levels did not correlate with body weight or BCS (data not shown).

Kommentiert [EZ4]: ….it is part of the results, hence they are somehow "shown". I would omit the brackets.

3.1 Feeding trial

All six cats successfully completed the study. Flavorless THBA powder could be easily administered with small amount of canned food. None of the cats showed any abnormalities related to THBA administration.

There were no changes in hematology results and biochemical profiles during the study (data not shown). Fasting blood glucose and serum level of fructosamine, insulin and THBA remained similar during the study (Table 2). During the ITT, measured $t_{1/2}$ and calculated K_{ITT} did not significantly differ between week zero and week eight (Table 2).

Kommentiert [EZ5]: No need to say…

Histological evaluation of adipose tissue was performed in five of six cats. Measurement of adipocyte size in one cat was impossible due to technical reasons. Median adipocyte size significantly decreased (P=0.04) from 43'168 μm^2 (range, 37'667–52'513) at week zero to 27'767 μm^2 (range, 25'535–37'221) at week eight (Fig. 1a and 1b).

Messenger RNA expression of MMP3, IL6 and TNFα in adipocytes significantly decreased (P=0.03), while mRNA expression of adiponectin significantly increased (P=0.03) at week eight compared to week zero. Messenger RNA expression of RORγ and leptin remained unchanged (Fig. 2).

3 Discussion

Kommentiert [EZ6]: There is a lot "We", "Our", "Us". I guess the discussion should be more impersonal. Some journals do not like when it is too personal.

In the first part of our study, we determined serum THBA in healthy and diabetic cats and tested if diabetic cats might have lower serum THBA compared to healthy cats. Feline endogenous THBA has not been described yet. In healthy adult humans endogenous THBA is present at minimal levels. High levels of THBA were reported in pregnant women and newborns (18,19,23). Evaluation of plasma THBA in metabolically healthy and prediabetic/diabetic obese and overweight humans showed negative correlation with body mass index, body fat percentage and adipocyte size. Hyperglycemic diabetic humans had significantly lower plasma THBA concentrations compared to non-diabetic individuals (C. Wolfrum, personal communication). Based on the results in humans, we assumed, that insulin resistant diabetic cats might have lower endogenous THBA compared to healthy. However, serum THBA concentrations in healthy and diabetic cats were similar and did not correlate with body weight and BCS. The reason for this finding is currently unknown. Type 2 diabetes mellitus significantly modifies bile acid levels, distribution and metabolism in humans (24-26). Plasma levels of cholic, deoxycholic, chenodeoxycholic bile acids are described to be negatively associated with insulin sensitivity (27). Knowledge about feline bile acid profile and the effect of type 2 diabetes mellitus on bile acid metabolism is limited and should be further evaluated in lean and obese healthy and diabetic cats.

Kommentiert [EZ7]: I would not write T2DM, for the above comment.
Actually, also "diabetes" without "T2" would be ideal, because in the same sentence you also mention healthy cats (non-diabetic).

In the second part of our study, we evaluated side effects of THBA and the effect of oral THBA supplementation on glucose homeostasis parameters, insulin sensitivity and adipocyte size in healthy normal weight cats. THBA was well tolerated, and was safe in cats. Oral THBA supplementation for eight weeks significantly reduced the size of feline subcutaneous adipocytes. Additionally, mRNA expression of MMP3 in adipocytes was significantly reduced, while mRNA expression of RORγ receptor remained unchanged. These data let us assume, that THBA might act on feline RORγ receptor, reduce mRNA expression of MMP3 and ensure the development of small adipocytes, as it was described in mice (14,16).

Adipocytes harvested from treated cats revealed decreased mRNA expression of TNFα and IL6 and increased mRNA expression of adiponectin. By contrast, mRNA expression of leptin remained unchanged. Increase of proinflammatory cytokines such as TNFα and IL6 in humans and cats are strongly related to IR (2,28-31), whereas adiponectin positively correlates with insulin sensitivity (2,32-34). Thus, our data indicate that THBA supplementation for eight weeks might improves insulin sensitivity of feline adipocytes. The reason for the unchanged mRNA expression of leptin is maintained body weight. Indeed, leptin increases proportionally to body fat mass (35,36).

Interestingly, we could not identify significant changes in fasting blood glucose, serum insulin and fructosamine at the end of the feeding trial. ITT results (K_{ITT} and t½) at the beginning and at the end of the study were similar to those previously reported in healthy normal weight cats (1). It might be due to the fact that insulin-mediated glucose disposal in lean body primarily occur in muscle and liver tissues (37). THBA improves insulin sensitivity of feline adipocytes. However, impact of adipose tissue on glucose metabolism and IR in lean feline body is low and increases with gaining body weight (2). Therefore, effect of THBA on glucose metabolism and IR should be further evaluated in obese non-diabetic and diabetic cats.

Our study has some limitations. Evaluation of serum THBA was performed in small number of healthy and diabetic cats and feeding trial did not include a control group. This study was designed as a preliminary evaluation of THBA's effects in cats. Major goal of the feeding trial was to evaluate side effects of THBA and provide basic information for further studies in obese non-diabetic and diabetic cats. Feline serum samples for THBA measurement were collected during routine check-up in the clinic and were therefore not standardized with regard to prior fasting, feeding time and amount, as well as quality of glycemic control (38,39).

4. Conclusion

THBA was detectable in feline serum and its concentration was similar in healthy and diabetic cats. In a feeding trial, synthetic THBA was well tolerated and is considered safe for use in cats. Oral THBA supplementation for eight weeks significantly reduced size and improved insulin sensitivity of subcutaneous adipocytes in healthy normal weight cats.

Kommentiert [EZ8]: Can we really say that? "insulin sensitivity of SC fat"? I would not link it to SC fat.. but remain more general.

Therefore, it might represent a new therapeutic agent for treatment and prevention of feline obesity-induced IR. Further studies are needed to evaluate the effect of THBA on adipocyte size, insulin sensitivity and glucose metabolism in obese and diabetic cats.

5. Acknowledgments

This work was partially supported by the Society of Comparative Endocrinology (Jack Oliver Graduate Research Award 2015).

6. Declaration of interest

THBA was provided by Prof. Christian Wolfrum.

7. Abbreviations

IR	insulin resistance
RORγ	retinoid related orphan receptor gamma
THBA	tretra-hydroxylated bile acid
MMP3	matrix metalloproteinase 3
ITT	insulin tolerance test
K_{ITT}	rate constant for the disappearance of glucose

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