Title: VY6, a β-lactoglobulin-derived peptide, altered metabolic lipid pathways in the zebra fish liver.

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Abstract Today enormous research efforts are focused on alleviating the massive, adverse effects of obesity. Short peptides are key targets for research as they can be generated from natural proteins, like milk. Here we conducted Trypsinogen digestion of Beta-lactoglobulin (β-Lg), the major mammalian milk protein, to release the hexamer VY6. It was assayed in vivo for its activities on lipid metabolism using zebra fish as a vertebrate model. Zebra fish juveniles were injected with two different doses of the peptide: 100 and 800µg g-1 fish and left for 5-days before sacrificing. Lipids measurements showed significant reduction in liver triglycerides and free cholesterol, as well as increased liver HDL cholesterol. Dose-dependent increases of mRNA levels of the genes coding for the enzymes acyl coenzyme A oxidase 1 (*acox1*) and Lipoprotein lipase (*lpl*) were also found. The whole results suggest significant anti-obesity activity of the β-Lg-derived VY6 peptide. Its use as a nutraceutical is discussed.

A. Introduction

More than 40 million children under 5 years old and more than 1.4 billion adults above 20 years old are suffering obesity[1,2]. The dimension of the problem is enormous and researchers and health agents are looking together for remedies to prevent and control the pandemics. The search of food components, preferably derived from natural products, able to decrease metabolic lipid contents and controlling cholesterol levels is therefore a priority.

Genetics has indeed a role in obesity. Among several enzymes responsible for lipids catabolism, acyl-coenzyme A oxidase 1 (Acox1) and lipoprotein lipase (Lpl) are of special importance. The product of the transcript *acox1* catabolizes both fatty acids and reactive oxygen species (ROS) in the peroxisomal β-oxidation process. In liver and adipose tissues, it is the first enzyme in such metabolic oxidation route[3,4]. Up-regulation of *acox1* gene expression is correlated with obesity-inhibitory treatments in the livers of different experimental models and at different stages of life, starting very early in the development[5,6]. Likewise, the enzyme produced by *lpl* transcript is a potent controller of lipids, chiefly acting as rate-limiting enzyme for the hydrolysis of triglycerides and being a potent anti-hypertriglyceridemia molecule[7]. Therefore, any food product able to activate these two genes is expected to contribute to control obesity.

β-lactoglobulin (β-lg) is one of the chief proteins that remain soluble within non-skimmed or skimmed milk[8]. β-lg-derived peptide VAGTWY (VY6) has already been assayed *in vitro* for different biological activities, and exhibits anti-diabetic[9], anti-bacterial[10,11], and ACE-inhibitory and opioid-like properties[12,13]. However, to date no assays have been reported for anti-obesity properties, neither in vertebrate models. VY6 showed some glucose regulatory capability in mice[14]. However, they administered whole β-lg hydrolysates, not the purified peptide. In fact, the utilization of zebra fish as an animal model to assay the aforementioned peptide bioactivities constitutes a novelty, since rats are normally the animal of choice[15,16,17,18]. Zebra fish is an animal model of choice for studies of human obesity because the metabolic routes that control the processes conducing to obesity in zebra fish are quite similar to the human's[19,20]. In this study, VY6 was obtained from β-lg by tryptic hydrolysis, and then isolated using a two-step process: ultrafiltration of the hydrolysate and semi-preparative RP-HPLC fractionation of the permeate. It is important to highlight that bioactivity assessments are usually performed using synthetic peptides, rather than directly purified peptides from the source of origin, as in the present case, as synthesized peptides lack functional inhibitors or activity-modifiers deliberately added from the original protein during the course of its purification for isolation of targeted peptides[21,22,23]. VY6 was subsequently assayed for its anti-obesity properties. I*n vivo* assays were performed using zebra fish (*Danio rerio*) as an animal model, focusing on lipid contents and in the modification of the *acox1* and *lpl* gene expression by reverse transcription-polymerase chain reaction (RT-PCR) technique that is used in several studies dealing with the effect of different nutraceuticals on physiological performance in human and experimental animals[24,25,26].

B. Materials and Methods

B.1. Production of the peptide VY6

The commercial substrate of Bovine β-lg (supplied by Davisco Foods International Inc., Le Sueur, MN, USA) contained 97.9 % protein (w/w) of which 91.5 % were β-lg, according to the manufacturer. Trypsin (T1426 from bovine pancreas TPCK treated, activity of ≥10000 units/mg protein), acetonitrile grade HPLC and TFA were purchased from Sigma-Aldrich (St. Louis, MO, USA), VWR (Barcelona, Spain) and Panreac (Barcelona, Spain), respectively. All other chemicals were of analytical grade.

Bovine β-lg solutions were prepared by solubilizing the protein powder in3 L of distilled water until a concentration of 30 g L^{-1} was reached. The pH and temperature values were set to 8 and 37 ºC respectively. Hydrolysis was carried out for 23h by adding trypsin at an enzyme:substrate ratio of 1:450 (w/w). The pH was kept constant with a pH-stat device model 842 Titrando (Metrohm Ion Analysis, Herisau, Switzerland), that added 1M NaOH when necessary. Reaction was stopped by lowering the pH with 0.1 M HCl, when the degree of hydrolysis (DH) reached the target value of 6.8 %. The calculation of the DH was based in the pH-stat method[27], using Eq. (1).

$$DH(\%) = \frac{B \cdot Nb}{\alpha \cdot MP \cdot hTOT} \times 100 \quad (1)$$

Where B (mL) is the volume of consumed base, N_B (mol L^{-1}) the normality of the base, M (g) the mass of protein, h_{TOT} the total number of peptide bonds in the substrate protein. This value was previously calculated by Cheison et al. (2010)[28] as 7.2 meqv g^{-1} protein for β-lg.α is the average degree of dissociation of α-amino groups in the hydrolysis system β-lg + Trypsin. In the present study, α was calculated according to Camacho et al. (2001)[29], and it took the value of 0.979 at pH 8.

For the membrane permeation experiments, the hydrolysates were first diluted in distilled water to a concentration of 15 g L^{-1}. The membrane used for the fractionation process, made of polyethersulfone (PES) with 5 kDa molecular weight cut off (MWCO) and 0.1 m^2 filtration area (Millipore, Billerica, MA, USA),was installed within a Pellicon 2 mini holder (Millipore, Billerica, MA, USA). The filtration conditions were pH 8, temperature 37 ºC and transmembrane pressure (TMP) 7.5×10^5 Pa. All membrane experiments were performed in duplicate.

Peptides present in the permeate stream were further separated by a Varian ProStarsemi-preparative RP-HPLC (Varian, Palo Alto, CA, USA), equipped with a fraction collector. Permeate samples were injected into a Dynamax Microsorb 300-8 C18 (250×21.4 mm, Varian, CA, USA) column, preceded by a guard Dynamax Microsorb C18 column (Varian, CA, USA). The system was run at ambient temperature and a flow rate of 10 mL min^{-1}, and light detection was set to 214 nm. Mobile phase A was composed of 0.1 % TFA (v/v) in MilliQ water (Millipore, Billerica, MA, USA) and mobile phase B was 0.1 % TFA (v/v) in acetonitrile. The gradient was: 5% B (0,1% TFA (v/v) in acetonitrile) for 8 min, 5 to 45 % B in 142 min, 45 to 80 % B in 20 min, 80 to 95 % B in 5 min and returning to the starting conditions in 1 min.

The collected fractions were analysed with a Voyager-DE STR matrix assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF/MS) (Applied Biosystems, Waltham, MS, USA). Sample preparation was performed with Zip-Tip pipette tips containing C18 reverse-phase media (Millipore, Billerica, MA, USA). A α-cyano-4-hydroxy-cinnamic acid (10mg mL^{-1} in 50 % Acetonitrile-0.1 % TFA) (Sigma, St. Louis, MO, USA) matrix was used. The MW range for the MS analysis was from 500 to 3500 Da, and for external calibration a MSCAL2 calibration kit (Sigma, USA) was used. Mass spectrometry acquisition was performed in reflectron mode and positive polarity, with an acceleration voltage of 25 kV. The spectra were processed with software Data Explorer (Applied Biosystems, Waltham, MS, USA). Peptide identification was carried out by MW, using the FindPept tool on the ExPASy server (http://web.expasy.org/findpept/).

The fractions were further analysed by direct-infusion electrospray mass spectrometry (ESI-MS/MS), so as to confirm the correct identification of peptide, using a quadrupole time-of-flight mass spectrometer equipped with an electrospray source (Qstar XL, Applied Biosystems, Waltham, MS, USA). The obtained MS/MS spectra were processed using Analyst QS 1.1 (Applied Biosystems, Waltham, MS, USA).

The peptide of interest (VY6) was stored until zebra fish tests using a Cryodos laboratory freeze-dryer (Telstar Industrial, S.L., Terrassa, Barcelona, Spain).

B.2. In vivo trial: zebra fish treatment, sampling and measurements

The experiments with living zebra fish followed the EU standards for animal experiments and were approved by the Committee of Ethics of the Asturias Principality as it is in the Directive of the University of Oviedo of 13 of February of 2014 (BOPA 26/02/2014).

Juvenile zebra fish *Danio rerio* were purchased from ZF Biolabs (Madrid, Spain), where they were raised under the minimum levels of consanguinity. Fifteen fish were acclimated for 14 days to normal, dechlorinated freshwater at ambient temperature (22 ºC) and normal summer light regime. They were fed with standard fishmeal for tropical aquarium fishes (Dajana Tropica Basic), containing 7 % crude oils and fat materials, one dose a day as recommended. Despite being the knowledge about the real requirements of nutrients lacking in zebra fish and many other ornamental fishes, the need for lipids should be adjusted since the low energy demands of the fish make them prawn to fatty deposition[30]. The experimental fish were divided at random in three groups of five fish of similar weights, ranging between 0.8796 ±0.096 and 1.145± 0.19 g. Two groups were treated with two different doses of VY6, and the control group was treated with water.

Fish were anaesthetized using sublethal 50 mg L^{-1} of Ethyl 3-aminobenzoate methanesulfonate (Tricaine, Santa Cruz Biotechnology, Cat. no. 10743661). Each fish was intraperitoneally-injected with 10 µL of one of the following doses: low (20 µg $µL^{-1}$: final *in vivo* injected dose=200 µg g^{-1} fish) or high (80 µg $µL^{-1}$: final *in vivo* injected dose=800 µg g^{-1} fish) of the peptide. In the control group the Zebra fish were injected with sterile water. For the injection 10 µL Hamilton syringes were used (Hamilton® GASTIGHT® syringe, cemented needle volume 10 µL, needle size 26s Ga-cone tip, Cat. no. 80039). After treatment the fish were kept in 20 L aquaria, under normal environmental and feeding conditions, in a closed-recirculation water system with mechanical filters for 7 days, renewing daily 20 % of tank waters.

For sampling, fish were anaesthetized by a lethal dose of Tricaine (100 mg L^{-1}). Each fish was dissected and the liver removed. Twenty mg of the liver tissue were placed in RNAse-

free 2 mL Eppendorf tube and immediately flash frozen in liquid nitrogen (N_2, 196 ºC) for later total RNA extraction, and the rest were kept in sterile 2 mL Eppendorf tube that was also flash frozen in liquid $N_2$for measurement of lipids. Both aliquots were maintained at –80 ºC until further analyses.

Total lipids were extracted from liver using the method of Folch et al. (1957)[31] with slight modifications. In brief, ultrafrozen livers (- 80 ºC) were individually homogenized in liquid N_2, then resuspended with strong vortexing in 500 µL of 2:1 chloroform:methanol mixture. The reconstituted samples were centrifuged at 10,000 g for 10 min. The supernatants were transferred to new 1.5 mL Eppendorf tubes, the process of adding 500 µL of 2:1 chloroform:methanol was repeated twice. Later on, 100 µL of 0.9 % NaCl were added to the supernatant and the tubes were thoroughly vortexed for strong mixing. The tubes were then centrifuged at 4.000 g for 30 min. The lower layer containing lipids was collected to a new 1.5 mL eppendorf tube that was incubated at 37 ºC for 2 days for Chloroform:Methanol mixture evaporation. The resulting lipids were reconstituted with 250 µL of 10 mM KH2PO4 a pH7, with strong vortex until complete lipids dissolution. In these samples, triglycerides, free cholesterol, and HDL cholesterol were measured using commercial kits from Spinreact (Cat. no. 1001310, 41035, and 10010196- respectively). KH_2PO_4 (10 mM, pH7) was used as negative control. External standard curve method was used for measurement of concentration in relation to the fluorescences emitted by lipids standards prepared as five, half-serial dilutions from each major standard provided with the lipids estimation kits. Extracted lipids samples and controls were applied in duplicates in a 96-well microplate for each treatment for measurement. Intra-specific errors were calculated as standard error of means of measurements between the duplicates of the same sample.

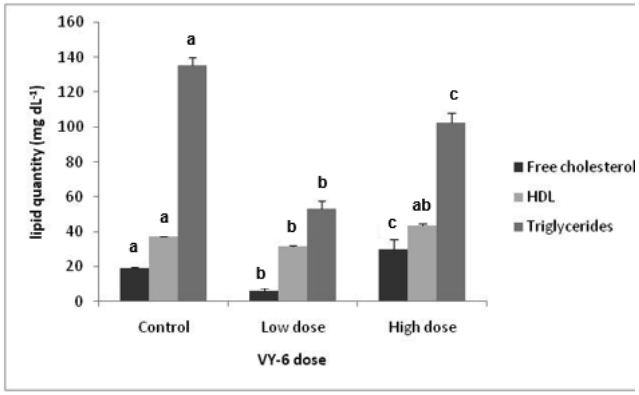
B.3. RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total liver RNA was extracted from each specimen using GeneMATRIX Universal RNA Purification Kit (Eurx, Cat. no. E3598) according to manufacturers´ instructions, reconstituting the tissue lysing buffer with 2-mercaptoethanol (Sigma-Aldrich, M3148-250ML). Extreme caution was taken to avoid introduction of RNAse into the working materials or samples. All glass materials used for preparation of reagents for RNA extraction were previously sterilized at 180ºC for 16 h. Working station, pipettes, centrifuges, and all other working materials were thoroughly cleaned with absolute ethyl alcohol. Diethylpyrocarbonate (DEPC, Merck, Cat. no. 8.41751.0005) was used for preparation of necessary dilutions of any material. After RNA extraction, the quality of RNA was checked using 2 %, RNase-free agarose gel (UltraPure™ Agarose, LifeTechnologies, Cat. no. 16500-100), prepared with 1X TBE (Eurx, Cat. no. E0230-01). 1 µL of RNA was loaded into the agarose gel using RNase-free gel-loading buffer (LifeTechnologies, Cat. no. AM8556.1). Success of extraction was confirmed by the presence of 2 bands, of the 28S rRNA and the 18S rRNA, with neither a smear or lower bands of degradation, nor high molecular weight band of genomic DNA. RNA quantity was determined through the spectrophotometric measurement of absorbance at A260/280 by BioPhotometer Plus (Eppendorf). Samples with A260/280 = 1.8-2 were considered adequate for cDNA synthesis. 500 ng of RNA from each liver samples were used for synthesis of cDNA, using EurxdART RT-PCR kit (E0802-01).

Semi-quantitative RT-PCR approach was used for assessing the effects of the injected hexamer on two enzymes´ genes related to lipid degradation and oxidation in liver, that are Peroxisomal acyl-coenzyme A oxidase 1 (*acox1*) and Lipoprotein lipase (*lpl*). The primers for *acox1* were: *acox1*Fw: 5´-ACAGCAGAGCAAGAGTAACG-3´ and *acox1*Rv: 5´-TGAAGGGCATAAAGCAGAGC-3´. The primers for *lpl* were: *lpl*Fw: 5´-CGCAGGAGCAGCAAGATG-3´ and *lpl*Rv: 5´-GTTCAAAGTAGGCATAATGTAGGG -3´. Beta actin (*actb*) was used as reference gene as its expression does not change under different chemical treatments to the zebra fish[32]. The primers used for *actb* amplification in liver cDNA were *β-act*Fw: 5´-CGAGCTGTCTTCCCATCCA-3´ and *β-act*Rv: 5´-TCACCAACGTAGCTGTCTTTCTG-3´[33,34,35]. All PCRs were carried out using thermal cycler Applied Biosystems Model 2720-2. The PCR mix contained in the previously mentioned RT-PCR kit, consisted of 1x PCR buffer, 0.2 mM dNTPs, 0.4 mM of each primer, and 2.5 units of *Opti-Taq*DNA polymerase. The PCR program consisted of an initial denaturation step at 95 ºC for 10 min, then 30 cycles of 95ºC for 30 s, 50 ºC for 30 s, and 30 s at 72 ºC for the extension. The annealing temperature (50ºC) was chosen according to preliminary optimization trials. Finally, PCR products were visualized in 2 % agarose gel stained by SimplySafe (Eurx, Cat. no. E4600-01) nucleic acids intercalating dye. The intensity of the resulting bands were measured using the program ImageJ[36]. The values of each band in the gel were normalized dividing the band intensity by the intensity of the band of the reference gene (*actb*) of the same sample.

B.4 Statistical analyses

For all measurements, the results were analyzed using one-way ANOVA in the program Statgraphics Centurion IX. Tukey HSD was used as post-hoc test to estimate statistical differences between groups of fish in pairwise tests. Differences were considered significant at $P<0.05$.



C. **Results**

C.1. VY6 production

As stated in section B.1, the identity of the peptide was confirmed by MALDI-TOF/MS and MS/MS. Additionally, it was characterised by RP-HPLC so as to assess the purity of the fraction. No additional peaks were found in the chromatogram, as it can be seen in Figure 1. The peptide was characterized by a retention time (RT) of 14.83 min. The National Center for Biotechnology Information (NCBI) database was searched for the bovine β-lg sequence, so as to confirm that VY6 sequence was within the protein sequence. VY6 corresponded to the 15^{th}-20^{th} amino acid residues.

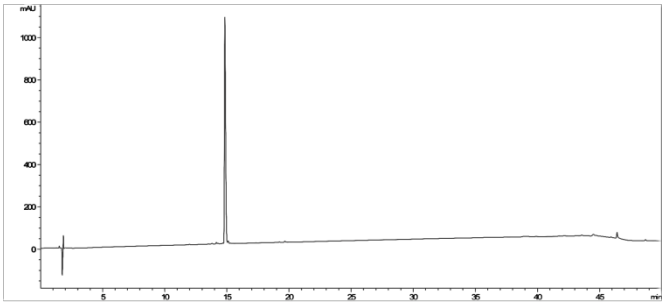


Fig. 1: RP-HPLC chromatographic profile of the semipreparative RP-HPLC isolated fraction identified as VY6 by MALDI-TOF/MS and MS/MS.

C.2. Effect of VY6 on zebra fish hepatic lipids

For all measurements, the ranges of linearity of standard curves covered adequately the concentrations used. r^2 in all cases ranged between 0.999-1. Moreover, very low intraspecific error were found between duplicates of each sample measure (1-3 x 10^{-3} for triglycerides and free cholesterol, and 4-5 x 10^{-3} for HDL) (see Table 1). Peptide injection induced significant reduction in the levels of triglycerides in liver (one-way ANOVA with F= 75.35, P<0.001; pairwise values in the Tukey's test are all significant, see Table 2) The decrease was more intense in response to the lower dose of the peptide injected (about one half of the control level; Figure 2), than with the high dose for which the reduction of triglycerides was significant (comparison control-high dose with a p-value of 0.007; Table 2). For the free cholesterol the F-value in a one-way ANOVA was 11.36 (P=0.009); however, the significant pairwise comparison was between the groups treated with the high and the low dose (Table 2), but not with the control that was intermediate between the two treated groups. A similar pattern was found for HDL, with the low-dose treatment exhibiting the lowest concentration and the control being intermediate (Figure 2). In this case all the pairwise comparisons were significant (Table 2 below) due to very low within-treatment variance.

Fig 2. Levels (mg dL^{-1}) of free cholesterol (x100), HDL/LDL cholesterol and triglycerides in zebra fish liver after treatment with VY6. Results are represented as average ± standard error of means. Different letters (a,b,c) above bars of each lipid category refer to significant differences among this lipid´s measured concentrations in response to injection with nil, low, and high doses of VY-6 (P<0.01).

Table 1. Values of regression coefficients (R^2), ranges tested for serial standards used for metabolites calculations, and intraspecific errors among duplicates of samples.

Lipids	Treatment groups	Intraspecific error	R^2	Linearity range
Triglycerides	Control	0.001	0.999	250-0.4 mg dL^{-1}
	Low dose	0.002		
	High dose	0.003		
Free colesterol	Control	0.001	0.999	250-0.4 mg dL^{-1}
	Low dose	0.002		
	High dose	0.003		
HDL	Control	0.004	0.999	42-0.34 mg dL^{-1}
	Low dose	0.005		
	High dose	0.004		

Table 2. Pairwise Tukey's test values for the differences between experimental groups in average of lipid concentrations, below diagonals. P-values: above diagonals.

Lipids	Treatment groups	Control	Low dose	High dose
Triglycerides	Control	-	0.0002	0.007
	Low dose	17.22	-	0.001
	High dose	6.721	10.5	-
Free cholesterol	Control	-	0.078	0.185
	Low dose	3.839	-	0.008
	High dose	2.879	6.718	-
HDL	Control	-	0.012	0.012
	Low dose	6.078	-	0.0005
	High dose	6.078	12.16	-

C.3. RT-PCR of *acox1* and *lpl* mRNA

The intraperitoneal injection of zebra fish with the hexamer peptide induced a dose-dependent increase of the expression of *acox1* and *lpl* genes, responsible for the degradation of fatty acids and triglycerides, respectively (Figure 3).

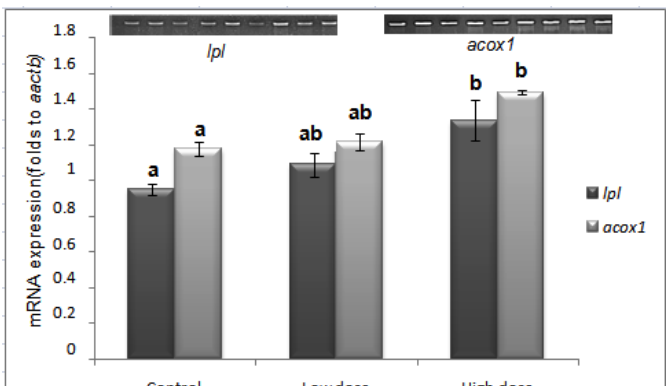


Fig 3. mRNA expression measured as mRNA levels of *lpl* and *acox1* transcripts in relation to *actb* as reference gene. Above: the RT-PCR patterns for *lpl* (left) and *acox1* (right) 3 control, 3 low dose, and 3 high dose samples. Results are represented as average ± standard error (bars). Different letters (a,b) above bars of each gene refer to significant differences among this gene´s measured expression in liver in response to injection with nil, low, and high doses of VY-6 (P<0.01).

The effect of the treatment was statistically significant for*acox1* (one-way ANOVA with F = 13.11, P = 0.002), and marginally significant for *lpl* (one-way ANOVA with F= 4.065 and P= 0.09). The dose-dependent effect is reflected in the second case in a significant difference between the control and the group treated with the higher dose (Table 3).

Table 3. Pairwise Tukey's test values for the differences between experimental group means of liver mRNA level of *lpl* (above diagonal) and *acox1* (below diagonal) genes. Significant values are marked as ** (p<0.01) and *** (p<0.001).

	Control	Low dose	High dose
Control	-	2.173	4.159 **
Low dose	0.642	-	2.016
High dose	5.943***	4.258**	-

D. Discussion

Zebra fish is considered a good model for metabolic assays of different human foods, drinks, drugs and nutraceuticals in general. For instance, it was used to test the efficiency of human nutraceuticals-delivering emulsions[37], algal polyphenols against stress[38], developmental anomalies resulting from inhibition of some essential mitochondrial energy production cycles enzymes by human drugs[39], green tea as obesity inhibitor[40], plant-derived flavonoids as Taxifolin against oxidative DNA damage[41], probiotics and their effects on the immune systems and also in reproduction[42,43], as well as many other, mostly human-related, nutraceuticals and drugs.

In the current trial, VY6 peptide enhanced the levels of HDL and reduced triglycerides efficiently. Enhancement also of mRNA levels of *lpl* and *acox1* enzymes in the zebra fish, our vertebrate experimental model, can refer to a positive role that VY6 plays as a nutraceutical, since these enzymes are active participants in fatty acids degradation. It is the first time to elucidate these physiological capabilities for the peptide VY6, what makes them indeed interesting and novel results. They strongly support the activity of VY6 peptide on lipid metabolism, and suggest this peptide may have an effect in preventing or combating obesity in vertebrates.

More in detail, *acox1* mediates both fatty acids and reactive oxygen species (ROS) metabolism in the peroxisomal β-oxidation process in liver and adipose tissues as the first enzyme in this metabolic oxidation route[3,4]. Up-regulation of a*cox1* gene expression correlates with obesity-inhibitory treatments in different experimental models. In general, its enhancement is always concomitant to anti-lipid deposition effects in almost all levels of vertebrates, yet we were the first to demonstrate it in zebra fish in response to β-lg-derived peptide VY6. For examples, *acox1* enhanced upon feeding mice on high fat diet mixed with concentrate of n-3 polyunsaturated fatty acids from fish oil, what eventually led to obesity reduction[44]. Moreover, *acox1* enhanced together with body fat reduction in obese zebra fish given green tea extract[5,6]. Broiler chickens given basal diet mixed with dehydroepiandrosterone (DHEA) shown enhance *acox1* levels[45]. Likewise, *lpl* is a potent controller for lipids, chiefly acting as rate-limiting enzyme for the hydrolysis of triglycerides and potent anti-hypertriglyceridemia[7]. Our results clearly demonstrated this anti-hypertriglyceridemic effect, with both concentrations tested of VY6 showing significant reduction in triglycerides. However, the more significant enhancement of *lpl* by the high dose than by the low dose may refer to VY6 capability to stimulate more *lpl* synthesis at the high dose. Adult liver in zebra fish is the organ where maximum *lpl* expression can be found, in comparison to other organs in the body[46]. Its expression in livers further enhances upon feeding animals on high fat diets[47,48].

Alongside with the increase of expression of the lipid-degrading enzyme genes, the anti-obesity effect of the assayed peptide is clearly revealed by significantly lower levels of hepatic ¨bad¨ cholesterol and significantly higher levels of the ¨good cholesterol¨ HDL. However, it seems that the low dose of the VY6 showed better overall results for lipids levels than the higher dose. For the insignificance of both *acox1* and *lpl* increase in response to the low dose of VY6, it should be taken into account that enzymes´ mRNA synthesis is often dependent upon the available quantity of the enzymes´ proteins. This means that if there is a high level of enzyme protein quantity and activity, the need for mRNA transcription for production of more enzyme units diminishes. We believe that this is the explanation of our case since ¨bad¨ lipids levels were much lower in response to VY-6 low dose than the high dose. Both *lpl* and *acox1* activities are governed via posttranslational mechanisms and the discrepancy for their mRNAs/proteins levels is known[49,50,51].

The VY6 peptide could be used as a nutraceutical if its effects on lipid metabolism are confirmed with further experiments with some mammalian models and even possibly with volunteers. Now it is known that animal and plant proteins are active source of nutraceuticals, which is a role extending far beyond their traditional role in nutrition. Food-derived peptides exhibit antimicrobial properties, blood pressure-lowering effects, cholesterol-lowering ability, antithrombotic and antioxidant activities, enhancement of immunomodulatory effects, inhibit cancer cell growth, enhance mineral uptake, diabetes, obesity, obesity-induced inflammation and exhibit potent opioid activities[52,53,54]. They can be effectively used then to reduce risk of disease or to enhance certain physiological functions.

Conclusions

The peptide VY6 derived from β-lg exhibited positive impacts on lipid deposition in the zebra fish liver. This, besides other previously demonstrated functions of that peptide, recommends VY6 as a promising, milk-derived nutraceutical that may exhibit positive effects against obesity and DPP-IV production.

Acknowledgements

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